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CYTOPLASMIC INCLUSIONS RESEMBLING GUARNIERI BODIES, AND OTHER PHENOMENA INDUCED BY MUTANTS OF THE VIRUS OF FOWLPOX *

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In the course of a study of induced mutation of fowlpox virus, it was observed that a variant strain of this virus, unlike field strains including its parent strain, was infectious for renal epithelium of newly hatched chicks. Direct injections into the kidney resulted in massive tubular epithelial hyperplasia superficially simulating neoplasm (Figs. 6 and 7). In the cytoplasm of the hyperplastic epithelial cells there appeared hitherto undescribed basophilic bodies reminiscent morphologically of a common form of Guarnieri body in vaccinial lesions, a form especially prominent in infected epithelial cells of rabbits. These structures were manifest in renal epithelium but were found also in cells of other lesions of the accompanying generalized infection, such as undifferentiated epithelium of the thymus, pulmonary alveolar ducts, biliary ducts, and the lympho-epithelial follicles of the organ of Fabricius (Fig. 8). Nevertheless, this alteration was not noticeable in all cells of a lesion; for example, they were not usually observed in the more differentiated cells of the hyperplastic renal tubules.

The basophilic inclusions, although developing separately, did not preclude the later appearance within the same cell of single or multiple lipid globules surrounded by an eosinophilic capsule (in hematoxylin and eosin stained preparations) which represented early stages in the formation of Bollinger bodies (Fig. 9). The Bollinger body consists of viral elementary bodies embedded in a lipoprotein matrix, and in well developed form is pathognomonic of fowlpox infection.[1]

The uniqueness of this abnormal reaction of cells to a mutant strain raised the question whether it represented the effect of a combination

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of fowlpox virus with an accidentally contaminating virus of vaccinia, in which each agent gave rise to a separate and specific reaction within the common host cell; or was the manifestation of a mutant which excited a response in some respects resembling that of vaccinia, in addition to one characteristic for fowlpox virus. It is well known that two different viruses can infect the same cell and each induce the formation of the inclusion characteristic of it.[2]

Investigation of this problem excluded the presence of vaccinia virus, and evoked evidence that one or more mutants of fowlpox virus may be responsible for the inclusions and for certain other cellular reactive phenomena of sufficient interest to report upon briefly. The altered virus was not infectious for rabbit skin or cornea; and its inoculation in chicks did not cause immunity to vaccinia virus or vice versa.

An Encephalitogenic Mutant of Fowlpox Virus. An infectious agent responsible for epidemic disease usually develops a characteristic pattern of pathogenesis in its definitive host. The pattern is rather strictly followed, for it is one that suffices to propagate and to disseminate the agent in a manner well adapted to its continued existence. This is true of the virus of epidemic fowlpox, which utilizes epithelium of the mouth and nasopharynx, as well as epithelium of the crop and skin, for reproduction and dissemination.

In the ordinary course of events the epithelial lesions predominate and are the only ones yet recognized in infected birds. On the other hand, persistence of virus in the blood after establishment of immunity not only makes it potentially available to blood-sucking insects, but indicates that it is harbored by other and probably different cells in the interior. The cytologic pattern is constant and consists of typical cellular hyperplasia and characteristic cytoplasmic inclusions (Bollinger bodies) which contain the virus.[3] Even though variant strains of fowlpox occur in nature (e.g., pigeon pox), the type of cytoplasmic inclusion remains the same.

It is unique in my experience to observe a mutation of a virus which causes the appearance of an entirely different inclusion, either alone or accompanied by the one from which it arose. The fact of such a change could be of significance in a study of the nature of the cellular responses to virus infection, and the inclusions of fowlpox are excellently adaptable to studies of such phenomena.

Although many viruses may undergo a variety of recognizable changes under laboratory conditions of propagation, a long experience with fowlpox led to the assumption of extraordinary stability, until experiments of G. J. Buddingh indicated otherwise.[4,5] He showed that

injection of a certain old laboratory strain into the brains of baby chicks regularly induced an encephalitis accompanied by opisthotonus, convulsions and death after several days. The cerebral lesion consisted of infection and hyperplasia of endothelial cells of capillaries, and of epithelium of the choroid plexus. When re-transplanted upon the chorioallantoic (c-a) membrane of developing chick embryos, a remarkable result ensued. Infected epithelium of both ectoderm and entoderm multiplied and dissociated so that the membranes became exfoliated and coated with what appeared to be white exudate. In the mesodermal tissue of the membrane, endothelial cells and fibroblasts became infected and likewise dissociated themselves to form enlarged spherical cells disposed in the fibrous meshes of this tissue. Cytoplasmic vacuoles surrounded by thin eosinophilic membranes appeared in the affected cells, but no basophilic bodies were noted. It appeared that after intracerebral inoculation a mutant predominated which was not recognized in the original field strain in the course of experimental infections. The mutant, in addition to its change of cytotropism, caused infected cells to become peculiarly dissociated from their normal tissue relationships. These observations were briefly recorded by Buddingh, but the matter was not immediately pursued. He succeeded in inducing the same changes with a second field strain, but in view of subsequent events I suspect this inoculum was contaminated with the first strain.

Several years later under the conviction that this phenomenon could be readily repeated, and probably with any field strain cultivated for a few generations in the c-a membrane, I undertook a repetition of Buddingh's experiments with several strains of fowlpox virus. In intervening years his original strains had been discarded.

Several months were spent in fruitless efforts to induce encephalitis by similar techniques, using the following strains of virus: (a) a c-a membrane adapted strain, passaged 113 times*; (b) a strain similarly passaged 38 times*; (c) a recently isolated field strain from a chicken, passaged 10 times in embryonic membranes within a few weeks*; (d) a field strain recently isolated from a turkey†; (e) a strain recently isolated from a chicken and established in membranes of chick embryos.†

In many attempts using each of these strains, there was no instance of encephalitis as described by Buddingh. Nor did any of these strains, cultivated for varying lengths of time on the chick membrane, cause a recognizable infection of endothelial cells, notwithstanding the occur-

* Supplied by Dr. F. R. Beaudette.
† Supplied by Dr. E. R. Doll.

rence of many other peculiar phenomena. The conclusion I reached was that Buddingh ran into a rare instance of a mutant capable of infecting capillary endothelium of brain and chorioallantoic membrane.

Other Mutants of Fowlpox Virus. Having failed to obtain an encephalitogenic mutant from any of several strains, an attempt was made to derive any recognizable mutations by subjecting a single field strain, recently isolated from chickens, to abnormal environments in the chicken host.

Material and Methods

A bacteriologically sterile strain of fowlpox virus (DV), derived from a recent outbreak in chickens and supplied by Doctor E. R. Doll of the University of Kentucky, was established by infecting the plucked scalp of two-day-old chicks, and harvesting the lesions after 7 days. A bacteria-free strain (DVM) was established by inoculating chorioallantoic membranes with .1 ml. of a 1:1,000 suspension (by volume) of infected epithelium in saline containing 500 units of penicillin per ml.

This strain was passed through 8 generations in chick membranes, each controlled for sterility by bacterial cultures. With the eighth passage (DVM_8) an intracerebral inoculation was made (DVM_8C_1), and the culture was recovered by inoculation of c-a membranes with brain material. Material from this passage was used for a second intracerebral passage in chicks 4 days old, and at the same time .5 ml. of the inoculum was injected into the occipital bone, because in Buddingh's experiments osteoblasts were infected by the intracranial injections. One of the latter chicks, showing fowlpox lesions in the skin of the scalp at the site of injection, was etherized; the inoculated piece of occiput was removed aseptically and ground in a mortar with a little saline. This was diluted to a 1:1,000 suspension by volume and used to inoculate 12-day-old membranes. Four days later one of these showed gray linear opacities. These were found by smears to contain no bacteria, but myriad elementary bodies (Morosow method[6]) and many free spherical epithelial cells (Figs. 1 to 5). The surface of the membrane was washed with 1 ml. of saline by means of a capillary pipette, and slightly cloudy gray fluid (.5 ml.) procured. This was used to inoculate several 12-day membranes to establish the second membranal passage from bone ($DVM_8C_1M_1B_1M_2$) and also .1 ml. was injected by means of a 1 cc. syringe through a small needle into the cancellous bone of the ilium to the right of the midline in the back. By accident some of this inoculum entered the cortex of the right kidney. Seven days later there was a fowlpox lesion in the skin at the site of injection. The chick was etherized, and the vertebrae and both pelvic bones at

the level of inoculation were removed with adherent kidney, and fixed in Zenker's solution. Infection of the kidney was recognized by examination of microscopic sections.

After recognition of the infectivity of this strain for renal cortical epithelium, the original strain ($DVM_8C_1M_1B_1M_2$) was again passed through the brain of a 4-day-old chick, and the virus, recovered by c-a membrane infection, was put through 4 additional membranal passages and then used to establish a line of renal virus. In subsequent experiments this was carried through 6 passages in renal tissue by successive injections through bones of the pelvis without intervening membranal inoculations.

At intervals these passage strains were injected into the brains of 2- to 4-day old chicks to test for the induction of encephalitis. In no instance did the Buddingh type of infection of the brain ensue, but always a local and systemic infection followed; this was usually fatal to the newly hatched chicks.

After the eighth membranal and one intracerebral passage, this strain inoculated upon the c-a membrane of 10-day embryos caused the same proliferation and spherical dissociation of ectodermal epithelium and fibroblasts as was observed with the mutant strain of Buddingh. Likewise, the cytoplasmic inclusions showed the same abnormality; that is, small lipid vacuoles surrounded by eosinophilic membranes were all embedded about the nucleus in what appeared to be a gelled state of the cytoplasm. The infected epithelial cells, placed upon a warm stage, exhibited active pseudopodal protrusions and retractions of ectoplasm, but no change in total position (Fig. 5). Rarely, a spherical infected cell lay within a large vacuole of another cell, and in this medium it, too, manifested pseudopodal activity. Pseudopods may be recognized in photographs of membranes inoculated with the altered virus.

Evidences of Mutation of Fowlpox Virus

After the first intracerebral passage, the lesion on c-a membranes showed characteristically altered gross and microscopic features. Grossly the membranes were soft, pliable, cloudy, and covered by a milky material simulating pus, in contrast to the firm, opaque, hyperplastic and infected epithelium which resulted from a standard strain of fowlpox virus (Figs. 1 to 4). This was a suspension of dissociated, spherical, pseudopodally active inclusion-containing epithelial cells. Microscopically it was evident also that fibroblasts contained lipid vacuoles within an acidophilic membrane, constituting an early form of Bollinger body. Endothelial cells were not infected and an inoculum from such membranes did not induce encephalitis.

When it was discovered that a kidney had become infected by accidental inoculation, a more complete study of the generalized infection which resulted from local or intravenous inoculations was undertaken. After 5 or 6 days, the infected chicks (2 to 6 days old at the time of inoculation) seemed droopy, and focal lesions appeared as usual about unfeathered parts, head, fauces, legs and feet. The eruptive lesions, however, were different in quality from those resulting from infection with a field strain; they rapidly vesiculated and ulcerated instead of forming warty growths. Ulcerative internal lesions were found in the mucosa of the crop and nasopharynx, and microscopically a peculiar affection of internal organs was observed. The thymic glands were slightly enlarged and yellow. They showed widespread necrosis and absorption of lymphocytes; and thymic epithelial cells were in great abundance relatively and apparently absolutely. They contained early vacuolated Bollinger bodies and also distinct basophilic cytoplasmic inclusions resembling Guarnieri bodies. These inclusions will be described more particularly as they appeared in the renal cells.

The flat epithelium of alveolar ducts of the lung was increased in thickness in focal areas. The cells revealed similar evidences of fowlpox infection and contained basophilic inclusions. The same was true of biliary capillary ducts. Even upon gross examination, pale, miliary lesions on the surface of the liver indicated the sites of fowlpox infection. Similar foci appeared in the cortices of the kidneys where infections evidently began within undifferentiated nephrons just beneath the capsule. Lesions in testes, ovaries and adrenals were also observed occasionally. Perhaps most interesting of all were the deep, indurated nodules that developed late in the disease about the ankle and wing joints and in the periosteum of the leg bones. These nodules measured up to .5 cm. in diameter, and the skin over them was movable. Histologically, they were formed by hyperplasia of infected periosteal osteoblasts, and gradually exhibited fibrosis. There was an associated periosteal osteogenesis. Beneath these nodules were often extensive infection of endosteal osteoblasts, osteocytes, chondroblasts and cartilage cells, and considerable zones of associated necrosis. Infected cells contained only the small Bollinger bodies (Figs. 10 and 11).

Previous experience with infection of chicks with field strains of fowlpox virus had disclosed none of the above lesions of internal organs or of bones and joints, but for the purpose of control, certain comparative inoculations into the kidney were made with the third generation of the same strain (DV_3), obtained before mutation.

Experiment to Test Infectiousness of a Field Strain of Fowlpox Virus for Renal Tissue Following Direct Inoculation of the Kidney

A fragment of frozen, infected c-a membrane (DVM_3), bacteriologically sterile, was ground and suspended 1 to 10 in saline and injected in .15 ml. quantities by needle and a 1 ml. syringe into the right kidneys of four 7-day chicks. Twelve days later all 4 chicks had early fowlpox lesions of the skin at the site of injection, about the mouth and eyes, and on the feet and legs. One chick, especially sick, died under ether anesthesia and was necropsied. Each of the remaining 3 was sacrificed for similar study at two-day intervals thereafter. Usual fowlpox lesions of the skin, nasal and oral mucosa, and the crop were present in typical form, but no infection was detected in the kidneys. In the right kidney were evidences of the distintegrated inoculum but no indication of fowlpox infection. No lesions were found in liver, spleen, lungs, thymus, endocrine glands or other organs.

In order to determine whether or not the mutant strain of fowlpox was infectious for the kidney without initial local damage, the virus was injected intravenously. Strain $DVM_8C_1M_1B_1M_4$ in 1:100 suspension of ground c-a membrane was injected into wing veins of six 7-day chicks in .1 cc. quantities. Twelve days later 4 chicks were alive. They showed receding ulcerative skin lesions about the head and on the legs and feet. In both kidneys of each of these chicks there were superficial, miliary, gray cortical lesions which microscopically proved to be foci of fowlpox infection, manifested by cellular hyperplasia. Many cells contained basophilic inclusions simulating Guarnieri bodies as well as vacuolated early Bollinger bodies. The two strains were thus strikingly different. Especial attention was paid to the basophilic inclusion which simulated the Guarnieri body, appearing in cells infected with the mutant strain.

Cytoplasmic Basophilic Inclusions Induced by a Mutant of Fowlpox Virus

These inclusions were especially prominent in large, pale, hyperplastic epithelial cells of infected renal tubules. Although they were found also in certain epithelium of other organs, they were larger and more intensely basophilic in the kidney. Most differentiated cells of hyperplastic tubules showed no inclusions, but when the affected epithelium became enlarged and pale, and tended to fill the tubular lumen, the first inclusions to appear were the basophilic ones often situated between the nucleus and the basal pole. The bodies varied in form;

some were round, others triangular in shape with the base next to the nucleus; still others were coarsely granular and formed less homogeneous masses. It seemed evident that the basophilic substance increased in volume after it first appeared, but with age and enlargement the inclusions became less homogeneous and less intensely stained with hematoxylin. Usually one or more vacuoles surrounded by acidophilic capsules appeared in the cytoplasm of the same cell, indicating the formation of Bollinger bodies. The two inclusions arose separately, but as they became larger the basophilic structures tended to associate with and even to incorporate the vacuolated bodies.

It did not appear that the basophilic structures were formed by agglomerations of smaller structures, nor was evidence found that they were related to nuclear extrusions, although opportunities for determining these points were limited. Feulgen reaction on sections of kidney and on smears of c-a dissociated epithelium fixed by ethyl alcohol showed no evidence of deoxyribose nucleic acid in any inclusions.

Discussion

There has been a long-standing controversy as to whether or not viral inclusions essentially represent colonies of the respective virus. This problem as applied to fowlpox was studied extensively by the present author and his associates several years ago with the conclusive demonstration that the Bollinger body consisted of infectious virus embedded in a lipoprotein matrix.[1] This was done by separating the inclusions *in toto* from the cells by means of trypsin, washing them individually until free of circumambient virus, and drying them on a slide until exploded by surface tension. By scraping off portions of a single dried inclusion and inoculating feather follicles with them, it was proved that each contained multiple infectious units associated with several thousand elementary bodies (stained by the Morosow technique[6]) embedded in a lipoprotein matrix. In the development of these intracytoplasmic acidophilic inclusions, no associated basophilic bodies or substances were observed.

The situation with respect to the composition of the Guarnieri body or bodies seems to be less well defined. The extensive observations of Bland and Robinow[7] led them to conclude that the cytoplasmic inclusions of vaccinia in epithelial cells of the rabbit's cornea essentially represented colonies of elementary bodies embedded in a matrix, thus justifying, as in the case of the Bollinger body, von Prowazek's term *Chlamydozoa*.

On the other hand, other investigations, including the recent study of Ryden and Randall,[8] have postulated the extrusion of Feulgen-positive

material from the nucleus. The authors stated that "the occurrence of intracytoplasmic inclusion bodies prior to detection of infectious progeny of the virus suggests that either the virus was immature when first visualized or that inclusion body formation preceded demonstrable viral replication." Although Feulgen-positive inclusion bodies were present 6 hours after inoculation of HeLa cell cultures, "infective virus was not detected in the cells from the fourth to the tenth hour after inoculation."

The basophilic bodies induced by mutant fowlpox virus which developed separately from and earlier than the acidophilic Bollinger bodies did not give a positive Feulgen reaction. However, the conclusion seems to be justified that they are not composed of mature elementary bodies, because within the same cell, acidophilic Bollinger bodies, known to consist of mature elementary bodies embedded in a lipoprotein matrix, regularly developed in the more advanced stages of cellular infection. There is no convincing morphologic evidence that the basophilic bodies participate in the formation of the larger Bollinger bodies. It is possible, however, that the reservoirs of basophilic material which appear in cells infected with the mutant strain are caused by an incomplete blocking of some process in the formation of mature elementary bodies. Thus, material of this nature, usually invisible or ordinarily not detected in cells infected with a field strain, now accumulates in relatively great mass. The formation of mature Bollinger bodies is correspondingly delayed in these infections with the mutant strain and never reaches full development comparable with that resulting from infection with the parent field strain. Whether or not the basophilic material represents a pool of nucleic acid, as suggested for the earlier inclusions of vaccinia, is not known. Since it did not give a positive Feulgen reaction, it is probably not deoxyribonucleic acid. It is, at any rate, a definite product of infection by a mutant fowlpox virus. A chemical analysis of the nucleic acid content of Bollinger bodies has not come to my attention. With the use of such a mutant of fowlpox virus, a study of the chain of events in the formation of mature virus could be undertaken from a chemical standpoint.

Recently Kato and his associates[9] have described two types of cytoplasmic inclusions in free cells in fluid from the peritoneal cavity of young chickens inoculated intraperitoneally with virus of fowlpox. They designated as type A the inclusion which corresponds to the Bollinger body, and as type B another cytoplasmic inclusion which they described as staining red by Giemsa's method. They found that type B inclusions gave a positive Feulgen reaction and that no type A inclusions appeared without the type B. They concluded that B inclu-

sions consisted of nucleoprotein which contributed to the formation of complete fowlpox virus.

With the information at hand, it appears that Kato's B inclusion differs from the basophilic inclusion which I have described. The former is acidophilic with Giemsa staining and does not stain at all in hematoxylin and eosin preparations, while the latter reacts strongly to hematoxylin and is basophilic with Giemsa stain.

In my own studies with vaccinia in the epithelium of the chorioallantoic membrane of chick embryos and in rabbit skin and cornea, it appears that the initial deeply basophilic paranuclear inclusion is the analogue of the similar inclusions of fowlpox under discussion. With advancing vaccinial infection in cells of the chorioallantois, the more amorphous cytoplasmic clumps or masses that develop in infection with strains of low virulence are acidophilic in reaction and, as suitable smears stained by the Morosow technique have shown, are composed for the most part of fully formed elementary bodies. I agree with those who hold that the basophilic paranuclear inclusion of vaccinia differs from more acidophilic structures that may develop later. The latter consist in large part of mature elementary bodies.

It seems likely from this study and the studies of Buddingh that the virus of fowlpox undergoes a number of mutations which determine cytotropism, such as ability to infect endothelial cells and renal epithelium. These two properties are distinct in that the latter does not confer ability to infect endothelium and thus to induce encephalitis. Likewise, the property causing dissociation of infected epithelial cells and that inducing the formation of basophilic inclusions might each be governed by specific mutants. It is of especial interest that at least some mutants of fowlpox virus may be recognized by the specific morphologic alterations which they induce. This is, so far as I know, a unique phenomenon for an animal virus and should be helpful in studying problems of viral mutation.

Summary

An experimentally induced mutant strain of fowlpox virus is described. This brings about lesions differing considerably from those caused by a classical field strain. Some of these lesions are briefly described. It is suspected that more than one mutant and perhaps several are involved. Attempts to derive a mutant strain infectious for endothelium and capable of inducing an encephalitis on that basis, as described by Buddingh, have not been successful. It seems likely that a specific mutant is responsible for this effect.

The mutant strain here described is capable of infecting immature

epithelium of renal tubules by direct intrarenal inoculation or by intravenous injection. The infection resulting from intrarenal injection can cause a remarkable local adenomatous hyperplasia which superficially simulates neoplasm.

In epithelial cells infected with the mutant strain, basophilic cytoplasmic inclusions resembling Guarnieri bodies of smallpox and vaccinia appear. These are described, and the hypothesis is advanced that they may represent pools of material accumulating in the cell as a result of a blockage of a series of reactions in which the substance is ordinarily used by the cell infected with a field strain. The chemical nature of this substance is not known, but it is suggested that it is analogous to the basophilic inclusions of vaccinia although a positive Feulgen reaction was not obtained.

REFERENCES

1. Woodruff, C. E., and Goodpasture, E. W. The infectivity of isolated inclusion bodies of fowl-pox. *Am. J. Path.*, 1929, **5**, 1–10.
2. Anderson, K. Dual virus infection of single cells. *Am. J. Path.*, 1942, **18**, 577–583.
3. Goodpasture, E. W. Virus Diseases of Fowls as Exemplified by Contagious Epithelioma (Fowl-pox) of Chickens and Pigeons. In: Filterable Viruses. Rivers, T. M. (ed.). Williams & Wilkins Co., Baltimore, 1928, Chapter VII, pp. 233–270.
4. Buddingh, G. J. A meningo-encephalitis in chicks produced by the intracerebral injection of fowl pox virus. *J. Exper. Med.*, 1938, **67**, 921–932.
5. Buddingh, G. J. A study of the behavior of fowlpox virus modified by intracerebral passage. *J. Exper. Med.*, 1938, **67**, 933–940.
6. Morosow, M. A. Die Färbung der Paschenschen Körperchen durch Versilberung. *Centralbl. f. Bakteriol.*, 1926, **100**, 385–387.
7. Bland, J. O. W., and Robinow, C. F. The inclusion bodies of vaccinia and their relationship to the elementary bodies studied in cultures of the rabbit's cornea. *J. Path. & Bact.*, 1939, **48**, 381–403.
8. Ryden, F. W., and Randall, C. C. The growth cycle of vaccinia in HeLa cells correlated with concurrent cellular changes. *Am. J. Path.*, 1957, **33**, 367–383.
9. Kato, S.; Hagiwara, K.; Baba, E.; Sato, Y., and Kamahora, J. Studies on the new inclusion bodies of fowlpox virus. *Virus*, 1955, **5**, 318–324.

[*Illustrations follow*]

LEGENDS FOR FIGURES

Except where indicated, photomicrographs were prepared from sections stained with hematoxylin and eosin.

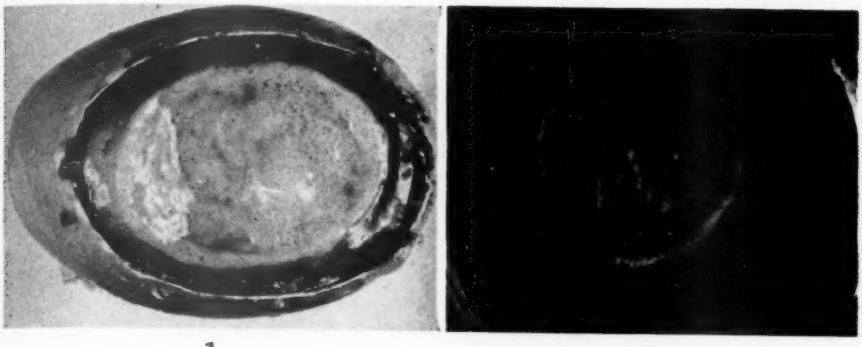
FIG. 1. Fowlpox lesion (field strain), 8 days' duration. The chorioallantoic membrane (CAM) is thick, firm, opaque, and slightly cystic. Slightly enlarged.

FIG. 2. Fowlpox lesion (mutant renal strain), 6 days. The CAM is congested, soft and pliable, and marked with opaque streaks of infected, dissociated hyperplastic epithelium grossly resembling pus.

FIG. 3. Ectodermal layer of CAM from Figure 1. Hyperplastic, coherent. × 25.

FIG. 4. Ectodermal layer from Figure 2. Hyperplastic epithelium is dissociated. × 25.

FIG. 5. Dissociated epithelial cells from CAM (Fig. 2). Note (a) early Bollinger bodies with vacuole and denser margin, and (b) pseudopods protruding from central mass of cytoplasm adjoining nucleus (arrow). × 1,000.



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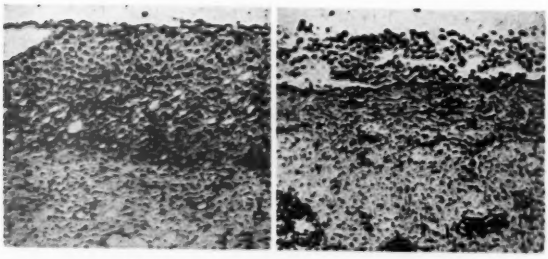

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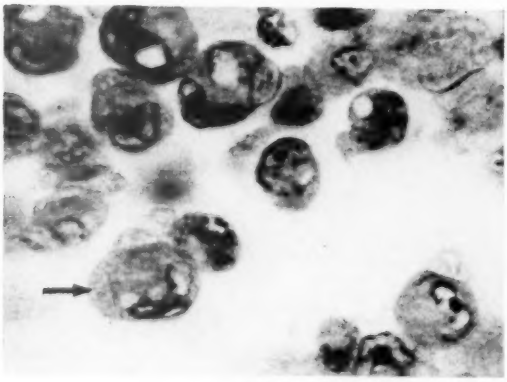

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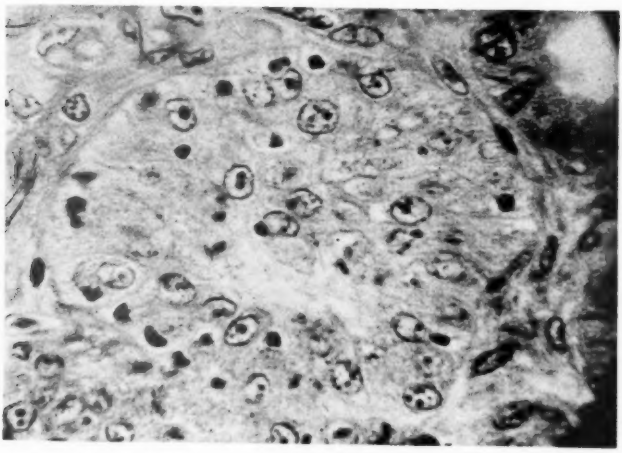
FIG. 8. Infected hyperplastic renal tubular epithelium from kidney in Figure 6. Note basophilic inclusions, irregular or crescent shaped, usually situated at basal pole of cell. No Bollinger bodies at this stage. × 425.

FIG. 9. Epithelial cells of another renal tubule from the same lesion, showing (arrows) both Bollinger bodies (vacuolated) and basophilic bodies in same cell. Mallory's aniline blue stain following Zenker fixation. × 850.



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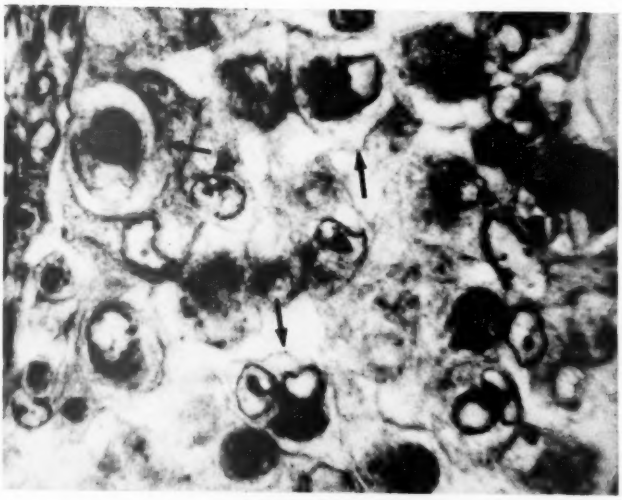

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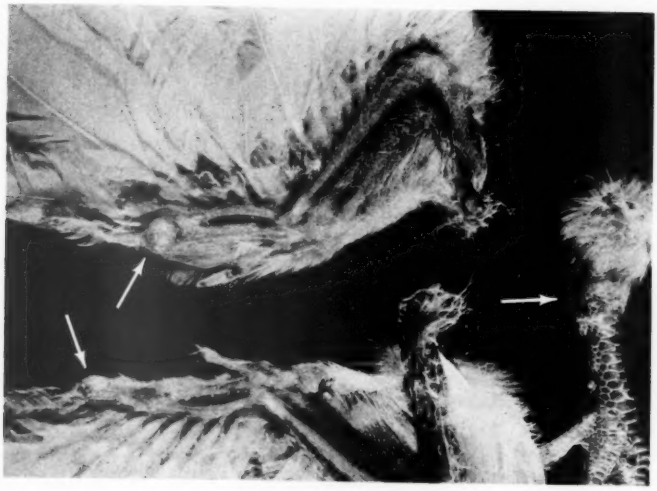
FIG. 10. Wings and foot of chick, showing periosteal nodules (arrows). × 2.

FIG. 11. Hyperplastic periosteal lesions of wing tip. Periosteal cells infected with renal mutant strain of fowlpox. × 30.



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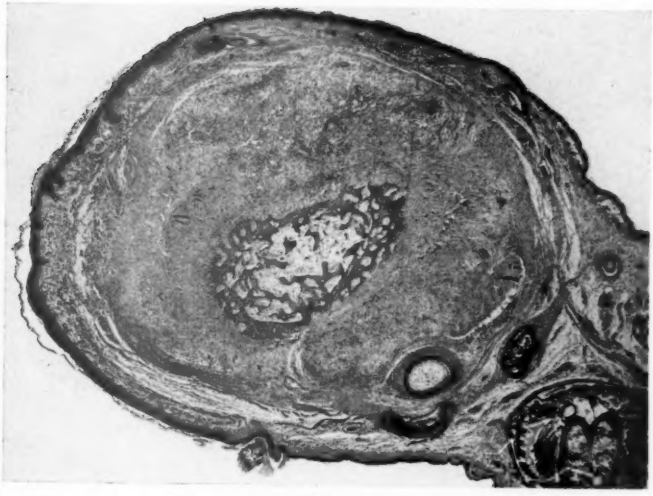

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THE SUPPRESSION OF LESIONS AND THE DEVELOPMENT OF IMMUNITY BY MEANS OF ANTIHYALURONIDASE IN VACCINIA AND FIBROMA INFECTIONS*

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During the course of studies on the Shope fibroma virus, it was observed that hyaluronidase greatly increased the size of the lesion produced by intradermal injection of the infectious agent; antihyaluronidase substances, however, tended to suppress them.[1] In addition, the antihyaluronidase was shown to affect the reaction of the host to the virus rather than to have a direct effect on the virus itself. It would be of interest to know whether the antihyaluronidase acts only to suppress the proliferation of fibroblasts or tumor cells, or is also capable of suppressing the reaction of the host to a virus that does not produce growths. Since the predominant ability of a virus such as vaccinia is to induce necrosis while that of the fibroma virus is to bring about proliferation of cells, it might be expected that the presence of antihyaluronidase would have no effect on the vaccinia lesion. On the other hand, if the lesions of vaccinia also are inhibited, the mode of action of the antihyaluronidase must be much more complex than simple suppression of growth. If lesions produced by vaccinia-antihyaluronidase mixtures should be negligible, as they are in the fibroma infection, and if animals surviving these lesions develop active immunity to re-infection, there are significant implications in the field of virus immunology in general.

The experiments to be described are directed toward the clarification of the following problems: Is the minimal fibroma produced by inoculation of the virus-antihyaluronidase mixture followed by active immunity? Does the combination of antihyaluronidase and vaccinia virus reduce the severity of the disease resulting from its inoculation? If this is the case, is immunity produced by the altered reaction, and if so, is it of as high a degree as in animals which have survived the fully developed disease?

MATERIAL

The antihyaluronidase preparations were provided by G. D. Searle and Company of Chicago and were identical with those used in our previous study. Their solution, SN 752, was used in the majority of the experiments; SN 80 B and SN 40 B were also used in a few. These

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substances are obtained from bovine and human umbilical cords.[2] They are not antibodies in an immunologic sense but are materials that prevent hyaluronidase from clearing the turbidity of a solution of hyaluronic acid *in vitro*. The hyaluronidase used in some of the earlier tests, the results of which are incorporated in the present study, was Alidase, a Searle commercial product.

Male rabbits of mixed breed, weighing approximately 3,000 gm., were used in all experiments.

The fibroma virus used in these experiments was the strain A virus originally isolated by Shope in 1932.[3] It was obtained from Doctor Shope in 1938 in the Laboratories of the Department of Animal Pathology of the Rockefeller Institute and has been maintained by serial skin passages in the rabbit in the laboratory of the senior author since then. During the interval, the reaction caused by the virus has changed; there is now a greater degree of interstitial mucin production and a smaller amount of cellular proliferation. The signs of disease in the rabbit, however, remain unchanged. The vaccinia virus was a highly virulent strain which had no proliferative effects. It was isolated by Pearce[4] from a spontaneously infected rabbit in 1938. Although the two viruses excite dissimilar reactions in the host tissues, they are of a comparable size.

Our experiences with each of the viruses and antihyaluronidase will be described separately.

Fibroma Virus

Methods

The inoculum of the Shope fibroma virus was made by grinding the skin tumor from a previous passage in a mortar with sterile sand and saline to make a 10 per cent suspension. This in turn was diluted to one tenth of its concentration before inoculation.

In the initial experiment designed to determine the inhibiting effect of antihyaluronidase, 20 rabbits were used. Six sites of injection were chosen on the skin of the shaved back of each. Into one of the 3 sites on each side, 0.5 cc. of a 50 per cent mixture of fibroma virus and antihyaluronidase was injected intradermally. One half cc. of a 50 per cent mixture of fibroma virus and hyaluronidase was injected into a second site on each side; and into the third site, 0.5 cc. of a 50 per cent mixture of fibroma virus and physiologic saline. To rule out any possible differences in local skin reactivity, the antihyaluronidase mixture was placed nearer the head on one side of the animal and nearer the tail on the other. This double set of injections made each of the 20 experimental animals its own control. Thus in the group of 20,

it was possible to achieve a total of 60 injections; 20 with the virus and hyaluronidase, 20 with the virus and antihyaluronidase, and 20 controls with virus and saline. The similarity of the lesions on the two sides of the same animal confirmed the validity of the results.

Another experiment was designed to compare the immunity produced by the virus-antihyaluronidase injection with that produced by the disease resulting from injection of virus alone. In this experiment also, 20 rabbits were used. Ten of these received an intradermal injection of 0.3 cc. of fibroma virus suspension mixed in a syringe with 0.4 cc. of antihyaluronidase in each of 6 sites on the shaven back. The other 10 received a similar injection at 6 sites, but saline was substituted in the syringe for the antihyaluronidase. In each case the amount of virus injected was the same. Five to 6 weeks later, long after their lesions had subsided, each of the 20 animals was bled to obtain serum for neutralizing antibody tests, and was thereafter reinoculated with proven potent fibroma virus.

Four tenths of a cc. of the serums of each of these rabbits was added to a suspension of fibroma virus of the same strength as that used previously (10 per cent) and also to dilutions of 10^{-1} and 10^{-2}. These mixtures of serum and virus were then injected intradermally into the backs of new rabbits; normal serum mixed with similar dilutions of virus was also introduced as a control. To assure adequate spacing on the rabbit's back, 4 experimental serums and a normal control serum were all that could be used on a single animal. Each rabbit had 15 injection sites, so that 5 rabbits sufficed to test the 20 experimental immune serums. There was thus a total of 60 sites for the 20 serums and virus dilutions and 15 sites for the normal serum and 3 virus dilutions. Since 2 rabbits were used for each set of 4 experimental serums and controls, there were 10 in all. These test animals were observed daily over a period of 2 weeks and then discarded.

Results

The results with the fibroma virus were uniform. Each of the 20 rabbits inoculated with the virus-saline and virus-antihyaluronidase mixture reacted in a similar way. At the site of the virus-saline mixture there was a large tumor. At the site of the virus-antihyaluronidase injection there was a small tumor or no tumor at all. The results of the virus-hyaluronidase injection will not be discussed here since they have been described elsewhere.[2,5-7] Since these reactions reached their height at approximately the seventh day, their size on this day has been recorded in Table I. The tumors were measured in their greatest diameter and in their least diameter, paralleling the skin surface, and

in their greatest elevation or thickness above it. The cubic volume was then arrived at by multiplying these 3 figures. In every instance the virus-induced fibroma lesion was inhibited by antihyaluronidase. The tumors sometimes failed to appear at all (Fig. 1) but as a rule were present but smaller than when uninhibited (Fig. 2). In addition they

TABLE I

The Effect of Antihyaluronidase on Growth of Virus-induced Fibroma Compared with That of a Saline-treated Control

	Fibroma virus + saline	Fibroma virus + antihyaluronidase
Number of lesions	40	40
Average greatest diameter (cm.)	2.5	1.2
Average least diameter (cm.)	2.3	1.0
Average thickness (cm.)	0.5	0.2
Average volume (cc.)	2.9	0.2

appeared later and regressed sooner than the controls produced by virus-saline mixture.

In the second part of the fibroma experiment, all 10 rabbits inoculated with fibroma virus and saline at 6 sites developed sizable tumors, approximately 2.5 cm. to 3 cm. in diameter. The 10 rabbits that were inoculated with fibroma virus and antihyaluronidase at 6 sites failed to develop tumors at some sites, and where tumors did appear, they were small, never being more than 1 cm. in diameter. A pair of these animals is shown for comparison in Figure 3. The serum of 19 of the rabbits completely neutralized the fibroma virus when mixed with it and injected into the skin of non-immune rabbits as described under "Methods." The serum of one of the rabbits that had received the virus-antihyaluronidase combination failed to neutralize the undiluted virus completely, but the tumor produced by this antiserum-virus mixture measured only 5 mm. in diameter. In the lesser concentrations (10^{-1} and 10^{-2}) there was complete neutralization, and no lesions appeared. Since, with this exception, neutralization occurred with the strongest virus solution, no difference in the level of antibody titer could be determined between the rabbits which received virus and saline and had large lesions and those that received virus and antihyaluronidase and had negligible lesions.

The 20 original experimental animals from which the neutralizing serums were obtained had acquired a firm, active immunity. Following re-inoculation with a 1 per cent concentration of fibroma virus, no lesion arose. Again, since there was no reaction whatsoever, no difference could be shown in the degree of immunity between the rabbits

receiving virus-antihyaluronidase which had shown inconsequential lesions and the virus-saline inoculated controls that had had the usual large tumors. Both groups were solidly immune to reinfection.

Vaccinia

Methods

The method used to determine the effect of antihyaluronidase on the vaccinia reaction differed only in detail from that described above. The inoculum was prepared using a 10 per cent suspension of vaccinia-infected testis from a previous passage. Six or occasionally 8 sites on the shaven backs of 6 rabbits were used. Into each of these sites 0.3 cc. of serial dilutions of the virus suspension mixed with 0.3 cc. of normal saline or with 0.3 cc. of antihyaluronidase were injected. No hyaluronidase was used in the vaccinia experiments, since its effect had already been well established.[1] The serial dilution method was used to determine a critical dosage at which vaccinia and saline would produce an extensive lesion, and vaccinia and antihyaluronidase would produce a much smaller lesion. In most animals the 10 per cent suspension diluted 10^{-6} (1 to 10,000,000) most closely approximated the dilution sought.

To control the factor of individual variation in the effect of antihyaluronidase on the vaccinia reaction, the lesions produced by the virus-antihyaluronidase mixture were compared with those produced by the virus combined with saline in the same animal. To do this, 10 additional subjects were used. Into these 10 rabbits, the same 10^{-6} dilution was injected intradermally at 6 sites on the shaven back. At 3 of these sites the inoculum was mixed with antihyaluronidase in equal proportions (0.3 cc. of each), and at the other 3, with saline solution (0.3 cc. of each). The two mixtures were alternated to allow for any possible difference in the reactivity of the skin at various levels. The animals were examined daily, and a comparison of the lesions produced by the virus with and without antihyaluronidase was recorded.

The other part of the vaccinia investigation was carried out in exactly the same way as the fibroma study, except that 20 pairs of rabbits were used. One of each pair received 0.3 cc. of vaccinia suspension diluted 10^{-6} mixed with 0.4 cc. of saline, and the other, 0.3 cc. of the 10^{-6} vaccinia suspension mixed with 0.4 cc. of antihyaluronidase at 6 intradermal sites on the shaven back. As a rule, the virus suspension and the saline or antihyaluronidase were drawn up into the same syringe and injected in that manner. In a few of the later animals a bleb was first raised in the skin by injecting 0.4 cc. of

saline or antihyaluronidase; 0.3 cc. of the suspension of virus was then injected into the bleb. The animals were examined daily and their temperatures recorded.

Again 6 to 8 weeks later, after all the lesions had disappeared, the surviving rabbits were bled for serum to be tested for neutralizing antibodies and were then vaccinated to test their own active immunity. This was done by injecting 0.3 cc. of a 1 per cent suspension of vaccinia-infected testicular tissue intradermally at 2 sites into the recovered animals and into 6 normal animals for a control.

In the determination of neutralizing antibody in the vaccinia experiments, a constant strength of virus was mixed with serial dilutions of the test serums. One cc. of a 10^{-5} dilution of virus was put into 9 cc. of the serum saline mixture, thus obtaining a final constant 10^{-6} dilution of virus against which the serums were tested. The serums of the rabbits which had had vaccinia virus and saline and those which had received vaccinia virus and antihyaluronidase, were compared with each other and with normal serum from untreated rabbits. All serums were diluted with saline 10^{-1} through 10^{-6}. Two normal rabbits received intradermal injections for each comparing titration, and were observed for 16 days.

Results

The results in the vaccinia experiments were positive, but they were somewhat less constant and more difficult to interpret than those in the fibroma series. In the titration experiment, at the sites where antihyaluronidase was combined with the vaccine virus the lesions appeared several days later than at those where virus alone was injected. In the higher dilutions, lesions frequently failed to appear at the antihyaluronidase site, and they were always smaller than those at the corresponding site into which virus alone had been injected.

In 2 of the 6 animals there was no appreciable difference in the extent of the lesion at the lower dilution. In general, however, there was no question that the antihyaluronidase tended to suppress the reaction of the host to the virus. The lesions in the 10 rabbits which had been inoculated at alternate sites with the same dilution (10^{-6}) of virus with and without antihyaluronidase were markedly different. Invariably the effect of antihyaluronidase was to delay the appearance and to suppress the severity of the reaction. This difference is well illustrated in Figure 4.

In the 20 pairs of rabbits in which one of each pair had been given antihyaluronidase with the virus, there was also a difference. However it was less striking than in the fibroma investigation. In no instance was the antihyaluronidase capable of completely suppressing

TABLE II

The Effect of Antihyaluronidase on the Reaction of the Rabbit to Vaccinia Virus

Rabbit no.		Local lesion: Day of onset	Local lesion: Severity	Highest temperature (°C.)	Days of fever
1	vaccinia–saline	2	+++	41.3	3
2	vaccinia–SN 752	6	+	40.5	3
3	vaccinia–saline	2	++++++	41.8	6
4	vaccinia–SN 752	6	++++	41.8	4
5	vaccinia–saline	4	+++	40.3	3
6	vaccinia–SN 752	6	++	39.4	1
7	vaccinia–saline	3	++	39.8	3
8	vaccinia–SN 752	4	+	39.9	1
9	vaccinia–saline	3	++++	41.0	6*
10	vaccinia–SN 752	5	+++	40.5	5†
11	vaccinia–saline	3	++++	40.7	6*
12	vaccinia–SN 752	5	+	40.0	4
13	vaccinia–saline	3	+++	40.4	5
14	vaccinia–SN 752	4	++	40.5	6
15	vaccinia–saline	4	+++	40.5	6*
16	vaccinia–SN 752	5	+	39.5	0
17	vaccinia–saline	3	+++	41.1	4
18	vaccinia–SN 752	5	+++	41.0	5
19	vaccinia–saline	4	++	40.0	3
20	vaccinia–SN 752	7	++	40.7	2
21	vaccinia–saline	3	+++++	41.5	6*
22	vaccinia–SN 752	4	+++	40.4	6*
23	vaccinia–saline	2	+++++++	41.3	7*
24	vaccinia–SN 752	5	++++	41.2	6
25	vaccinia–saline	3	++++	40.6	5*
26	vaccinia–SN 40B	7	++	40.3	2
27	vaccinia–saline	3	+++	41.3	5§
28	vaccinia–SN 752	7	+	41.2	6*
29	vaccinia–saline	3	++	40.4	6
30	vaccinia–SN 40B	7	+	41.1	3
31	vaccinia–saline	4	++++	41.4	4*
32	vaccinia–SN 80B	7	++	41.2	5*
33	vaccinia–saline	5	+++++	41.2	5*
34	vaccinia–SN 80B	7	++	41.4	3
35	vaccinia–saline	5	+++++	41.0	3§
36	vaccinia–SN 80B	0	0	39.5	0
37	vaccinia–saline	4	+++++	41.4	5*
38	vaccinia–SN 80B	7	++++	41.5	4*
39	vaccinia–saline	4	+++++	41.4	5*
40	vaccinia–SN 80B	7	+	40.2	3
Average	vaccinia–saline	3.35	3.7+	41.0	5.3
	vaccinia–antihyaluronidase	5.65	1.9+	40.0	3.4

* Generalized pox.

† This animal died but not with generalized pox. Cause of death could not be determined.

§ Generalized pox and death.

\+ — erythematous lesion 1 cm. or less in diameter.

++ — erythematous lesion 1 to 2 cm. in diameter.

+++ — erythematous lesion 2 to 3 cm. in diameter.

++++ — hemorrhagic lesion under 3 cm. in diameter.

+++++ and +++++— necrotic, sloughing hemorrhagic lesion over 3 cm. in diameter.

SN 752, SN 80B and SN 40B: preparations of antihyaluronidase.

the lesion. In 8 of the 20 antihyaluronidase treated animals the lesions were minimal, but in only 3 was the maximum temperature under 40° C. Eight rabbits had mild lesions as compared with the virus-saline inoculated controls, but the disease in the remaining 4 was comparable in severity with that in the vaccinia-saline group. In general the lesions in the controls that had no antihyaluronidase were appreciably larger. Another significant difference between the two groups was that only 3 of the antihyaluronidase-treated animals had multiple lesions, whereas 13 of the vaccinia-saline control group developed multiple pox, indicating generalization and severity of the disease. These differences are shown in Figures 5 and 6 and in Table II. The averages at the bottom of the table indicate a trend, but of course it is the difference between the individuals in each pair inoculated with exactly the same amount of virus that is significant.

In addition to the differences in the extent and severity of the lesions produced by the virus alone and combined with antihyaluronidase, there was again—as in the earlier experiment in which the same rabbit received both vaccine-antihyaluronidase and vaccine-saline at different sites—an appreciable difference in the time of appearance of the lesions. The vaccinia-saline induced lesions developed earlier and persisted longer. The animals inoculated with vaccinia-antihyaluronidase developed lesions much more slowly and recovered about the same time as their partners, so that although the time from inoculation to recovery was the same in them, the course of the disease was actually considerably shorter. The pair of rabbits numbered 25 and 26 was an example of this. Rabbit number 25 had fever and large lesions on the fourth day. On the seventh day the temperature was 41.2°C. and the lesions were 4 +. Fever over 40°C. and 4 + lesions continued for the next 5 days before any regression was seen. Number 26, on the other hand, first showed a minimal lesion on the seventh day and did not develop its maximum 2 + lesion and temperature of 40.3°C. until the eighth day. The lesions began to regress almost at once and by the twelfth day were disappearing. Number 25 had generalized pox; number 26, protected by antihyaluronidase, did not.

Of the 20 rabbits inoculated with vaccinia alone, 18 survived, 2 having died of generalized vaccinia, or rabbit pox. Of the 20 rabbits inoculated with the combination of vaccinia virus and antihyaluronidase, 19 survived, one having died of an unidentified disease that was not vaccinia. The 37 survivors and the 6 animals from which normal serum had been drawn for the antibody titration experiments were inoculated with a 1 per cent suspension of vaccinia-infected testis. The survivors were all solidly immune. Some developed a small red

papule, others had no reaction. No difference could be detected in the degree of immunity of those that had had antihyaluronidase combined with the initial virus infection and those that had not. Four of the 6 control animals died with generalized pox; the remaining 2 recovered from prostrating vaccinia.

The serums of the 19 surviving rabbits which had been inoculated with the vaccinia-antihyaluronidase mixture and had experienced a relatively mild disease had as much neutralizing antibody as the 18 surviving animals that had not had antihyaluronidase in their inoculums and had gone through the usual severe course of vaccinia. The serums of both groups protected completely through the 10^{-2} dilution. In the higher dilutions there was only partial protection, but it was not constantly greater in either group. Three examples of these comparative titrations are shown in Table III.

TABLE III

Neutralizing Antibody Titers in Serums of Rabbits Inoculated with Vaccinia Virus, Vaccinia Virus Combined with Antihyaluronidase, and Normal Rabbit Serums

Rabbit serums	Serum dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
In the skin of rabbit #80						
30 (vaccinia–antihyaluronidase)	o	o	+	+++	+++	+++
Normal	++	++	+++	+++	+++	+++
21 (vaccinia–saline)	o	o	+	+	+++	+++
In the skin of rabbit #81						
30 (vaccinia–antihyaluronidase)	o	o	++	++++	++++	++++
Normal	+	++++	++++	++++	++++	++++
21 (vaccinia–saline)	o	o	+	+++	+++	+++
In the skin of rabbit #82						
26 (vaccinia–antihyaluronidase)	o	o	+	+	++++	++++
Normal	++	++	+++	++++	++++	++++
29 (vaccinia–saline)	o	o	o	++	++++	++++

\+ — erythematous lesion 1 cm. or less in diameter.
++ — erythematous lesion 1 to 2 cm. in diameter.
+++ — erythematous lesion 2 to 3 cm. in diameter.
++++ — hemorrhagic lesion under 3 cm. in diameter.

DISCUSSION

It has been demonstrated that the simultaneous injection of an antihyaluronidase substance with either the Shope fibroma or vaccinia viruses had an effect on the reaction of the host to its infection. This was most striking in the case of the fibroma virus. The outward evidence of infection with this virus was often absent when antihyaluronidase was present in the inoculum, and at other times the lesion

was insignificant. Nevertheless the animals which had failed to develop a lesion following injection of the virus and those in which the lesion was extremely small were, by our methods of testing, as immune to re-infection as were those that had had large tumors. Therefore, as far as the fibroma virus is concerned, this would seem to be an easy, certain, and safe method of immunization.

In vaccinia the injection of antihyaluronidase also had a demonstrable effect. Sixteen of the 20 animals so treated had only mild or minimal vaccinia and yet developed strong immunity. Since there were 4 rabbits in which the lesions were severe and the disease systemic, this technique does not yet provide an entirely satisfactory method of immunization against vaccinia. Nevertheless, the fact that an antihyaluronidase-virus combination produces a lesion and discomfort does not mean in general that this may not be a practical method of immunization.

The mode of action of the antihyaluronidase substance remains obscure. That it does not simply inactivate or kill the virus as would heat or carbolic acid has been demonstrated in an earlier paper.[1] A mixture of antihyaluronidase and fibroma virus kept in the refrigerator overnight produced only a small fibroma, as would be expected, but when hyaluronidase was added to this combination, the antihyaluronidase substance was apparently neutralized, since the lesion then produced by the 3-fold mixture was as large as that produced when virus alone was injected.

Summary

Antihyaluronidase when injected in combination with the fibroma virus prevented or greatly diminished the development of growths of the characteristic lesion. Rabbits so treated had a high degree of immunity as measured both by active immunity to reinfection and by the level of neutralizing antibody in the blood serum.

Antihyaluronidase when combined with vaccinia virus in most instances appreciably diminished the severity of the resulting vaccinia. Animals recovering from this milder disease were immune to further inoculation and had circulating antibodies in their blood in a titer as high as that in rabbits which had recovered from the unsuppressed disease.

The ability of the antihyaluronidase substances to suppress the lesions and ameliorate the disease produced by these two viruses, while still not interfering with the development of firm, active immunity, indicates the possible usefulness of these substances in the prophylaxis of virus disease in general.

REFERENCES

1. Pearce, J. M., and La Sorte, A. F. Effects of an antihyaluronidase substance and of hyaluronidase on growth of virus-induced fibromas. *Proc. Soc. Exper. Biol. & Med.*, 1954, **86**, 573–577.
2. Hadidian, Z., and Pirie, N. W. The preparation and some properties of hyaluronic acid from human umbilical cord. *Biochem. J.*, 1948, **42**, 260–265.
3. Shope, R. E. A transmissible tumor-like condition in rabbits. *J. Exper. Med.*, 1932, **56**, 793–802.
4. Pearce, J. M. The persistence of vaccine virus in rabbits immunized to vaccinia by a previous infection and the relationship of immunity to latent virus. *J. Infect. Dis.*, 1940, **66**, 130–137.
5. Duran-Reynals, F. Exaltation de l'activité du virus vaccinal par les entraits de certains organes. *Compt. rend. Soc. de biol.*, 1928, **99**, 6–7.
6. Sprunt, D. H. The effect of the virus:host cell relationship on infection with vaccinia. *J. Exper. Med.*, 1941, **74**, 81–89.
7. Sprunt, D. H. The ground substance in infection. In: The Ground Substance of the Mesenchyme and Hyaluronidase. Duran-Reynals, F. (cons. ed.). *Ann. New York Acad. Sc.*, 1950, **52**(7), 1052–1060.

[*Illustrations follow*]

LEGENDS FOR FIGURES

FIGS. 1 and 2. The suppression by antihyaluronidase of the reaction to fibroma virus. The two sites of injection of antihyaluronidase and virus are indicated by the arrows. At the other sites where antihyaluronidase was not used, large tumors are obvious. In Figure 1 there is no reaction. In Figure 2 the reaction is visible but small.

FIG. 3. The upper of the two rabbits was inoculated with fibroma virus-saline mixture and as a result has 6 large lesions. The lower rabbit received the same amount of virus mixed with antihyaluronidase and has barely visible lesions.

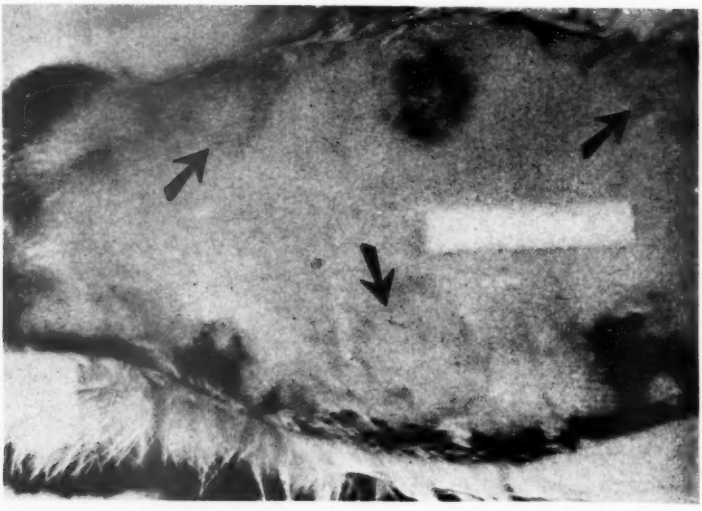
1

2

3

FIG. 4. Six sites of vaccinia virus injection. At the 3 indicated by the arrows, antihyaluronidase was mixed with the inoculum. Where virus alone was injected, the 3 necrotizing, hemorrhagic lesions are obvious.

FIG. 5. The upper of these two rabbits was injected with vaccinia virus and antihyaluronidase on the back, the lower with vaccinia virus and saline. In addition to the large hemorrhagic and necrotic lesions in the lower animal at the sites of injection, there is generalized pox as indicated by the many smaller hemorrhagic lesions. The upper animal has negligible lesions and no generalized pox. The plaque-like elevations of skin on this animal (no. 1605) are not lesions but are the normal variations in thickness and piliation that occur in the skin of some rabbits.



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5

PROTEIN IN VACCINIAL LESIONS REVEALED BY OXIDIZED TANNIN-AZO (OTA) METHOD*

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The persistence of protein in dead and dying cells is a notable feature of necrotic tissues.[1-3] But if necrosis is preceded by cellular swelling, very substantial quantities of protein may be lost from the injured cells.[2-5] Epidermal cells in the skin frequently become swollen and ballooned when they are killed by infection with dermotropic viruses such as vaccinia.[6] The distribution of epidermal protein was therefore studied in vaccinial lesions of the skin in order to determine whether protein was retained or lost in cells damaged by the virus. For this purpose the oxidized tannin-azo (OTA) technique,[7] and the tetrazo method of Danielli,[8] were used as methods of assessing the protein content.

MATERIAL AND METHODS

Rabbits aged 1 to 5 days were used. The abdominal skin in these young animals has so little hair that depilation is not required before vaccination. The skin of the abdomen was cleaned with ethanol, and vaccine lymph (Lister Institute, issued for vaccination) was applied as a longitudinal drop a few millimeters to one side of the umbilicus. Longitudinal linear scarifications of varying length up to 15 mm. were then made by sterile needles through the lymph. Scarification was deliberately of insufficient depth to draw blood. The lymph was rubbed into the scratches with the side of the needle. One, two or three such scratches were made on each animal. Slightly crusted, elevated lesions developed in 3 or 4 days.

The animals were sacrificed on the third or fourth day after vaccination. The raised vesicular lesions, with adjacent skin, were excised and fixed by immersion in Carnoy's fluid for two hours. In most of the specimens the subcutaneous fat was stripped away before fixation. The fixed tissues were embedded, and sections cut and affixed to slides as described elsewhere.[7] The sections were cut at right angles to the surface of the skin; unaffected skin in addition to the vaccinial vesiculation was included in each of the sections studied.

Sections of vaccinial lesions from 8 animals were stained by Harris' hematoxylin and aqueous eosin. The oxidized tannin-azo (OTA) method as described by Dixon[7] was also utilized. This method detects the basophilic, tannophilic groups in the tissue proteins. The sections

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were affixed to slides by incubation in a hot air oven at 60°C. overnight without the application of any adhesive. After removal of wax, the sections were then immersed in tannic acid HCl, oxidized with periodic acid, and finally coupled with diazotized o-dianisidine.* In this manner the tannic acid bound by the proteins in the tissues was converted into an intensely colored azo dye, thus locating the site of tannophilic protein. Control sections not oxidized with periodic acid between tanning and coupling were also examined in each tissue studied. Without oxidation the bound tannic acid gave only a feeble color on coupling. In sections of two of the specimens the tannophilic groups were also masked by pretreatment with formaldehyde before applying the OTA method.

The tetrazo method of Danielli[8] was also employed, using the borate-buffer technique of Dixon[9] as modified by Dixon and McCullagh.[5] This method reveals the location of tyrosine, tryptophan and histidine in the tissue proteins. Here, too, heating at 60°C. overnight, without adhesive, fixed the sections to the slides. Sections from lesions in 3 of the animals were examined by the Feulgen method, following the technique outlined by Dixon[3] except that incubation in Schiff's reagent lasted for 1.45 hours. In addition the tanno-ferric method described by Salazar[10] was applied, using the technique outlined by Dixon.[11]

Observations

Elevated regions of thickening and vesiculation developed in the skin of every animal vaccinated which remained alive till the third day after vaccination (15 of 17). In one rabbit the lesion appeared to be traumatized; this specimen was discarded. The lesions were usually elongated and extended along the linear scarifications. In a few cases infection was not so extensive, and circular papules were formed.

The structure and disposition of the lesions conformed to the classical descriptions of cutaneous vaccinia given by Unna[6] and later by Tyzzer,[12] Hammerschmidt,[13] and Amies.[14] The malpighian layer of the normal abdominal epidermis of young rabbits is only about 5 cells in thickness (Fig. 1). At the margins of the lesions, the cells increased both in size and number (Fig. 2), causing very substantial epidermal thickening (Fig. 3); mitotic figures were frequently visible. In the more central parts of the lesions, cellular vacuolation *("ballonnirende colliquation")* was prominent. The swollen spongiose cells

* Brentamine fast blue B salt, used for preparing diazotized o-dianisidine employed in the OTA method, was provided by Imperial Chemical Industries Ltd. (Dyestuffs Division).

ultimately degenerated into vesicles which were later invaded by neutrophils (Fig. 4). Some of the epidermal cells, however, showed coagulative necrosis and became eosinophilic without swelling or vacuolation. Even near regions of pronounced vacuolation the deeper epidermis remained at least partially intact. Figure 4 shows a group of such persistent cells beneath vacuolated necrotic remnants. These cells, although not vacuolated, frequently contained conspicuous cytoplasmic inclusion bodies. The *stratum corneum* persisted as a superficial covering to the vesicles.

Cytoplasmic inclusion bodies were seen in every vaccinial lesion examined. The oil immersion objective greatly aided in their detection. In the zone of proliferation, at the periphery of the lesions, increase in nucleolar size[13] was one of the first signs of abnormality in the epidermal elements. The inner cells of this zone usually showed prominent inclusion bodies (Figs. 2 and 12). In this location the inclusion bodies were round and discrete, and usually occurred singly in each affected cell. In the more central parts of the lesions the inclusions were less regular in size and shape,[13,14] and on occasion several were present in each cell. Indeed, careful scrutiny revealed aggregates of inclusion substance forming the bulk of the cytoplasm in many of these cells (Figs. 4 and 14). Aggregates were especially prominent in the cells of hair follicles which were occasionally loaded with granular masses of inclusions.[14] The inclusion bodies were well stained by aqueous eosin and were also intensely colored by the special stains for protein.

Protein in Unaffected Epidermis

Tanned sections, after oxidation with periodic acid, were stained salmon red on coupling with diazotized o-dianisidine.[7] The cells of the malpighian layer contained abundant protein stainable by this method; their cytoplasm and cell membranes were intensely colored although their nuclei were less strongly stained (Fig. 1). As the cells became cornified, their tannophilic capacity diminished; the cells of the *stratum corneum* were thus only tinged a faint yellow by the OTA method, while the hard keratin of hair was scarcely colored at all.

Control sections, not oxidized with periodic acid after tanning, were only very faintly colored by subsequent treatment with diazotized o-dianisidine. Moreover, immersion in aqueous formaldehyde overnight effectively masked the basic amino groups in the epidermal proteins which were responsible for binding tannin; after pretreatment with formaldehyde, sections of the skin were only faintly colored by the OTA method.

Epidermal protein was also conspicuous in sections stained by the tetrazo method. With this technique the *stratum corneum* and the cells of the malpighian layer were stained dark red. The hard keratin of hair was, however, not colored except for the fine scales of the cuticle which stained intensely.

Protein Content of Infected Cells

In the zone of proliferation at the margins of vaccinial lesions, the epidermal cells and enlarged nucleoli were strongly colored by the OTA method (Fig. 2). Inclusion bodies, intensely colored by OTA, were also visible in the cytoplasm of many of the cells (Fig. 2). A major portion of the total protein of the cytoplasm was contained in the inclusion bodies.

None of the constituents of the infected cells stained more than very faintly in control sections, which had not been oxidized by periodic acid between tanning and coupling with diazotized o-dianisidine. Pretreatment with formaldehyde also effectively inhibited the development of color when the OTA technique was applied.

The ballooned cells in the epidermal lesions were found to have lost most of their cytoplasmic protein when stained by the OTA or tetrazo techniques. As swelling and vacuolation proceeded, the content of protein diminished. Figures 1, 2, 5 and 6 illustrate a sequence of changes from normal skin outside a lesion to epidermal vacuolation within it. At the margin of the lesion in the zone of epidermal proliferation, many of the cells contained intensely stained inclusion bodies (Fig. 2). Despite swelling with loss of protein from the ballooned cells, some of the epidermal elements still retained substantial quantities of protein which was partly located in inclusion bodies (Fig. 5). With continued progression of the lesion, there was conspicuous vacuolation and loss of protein from many of the epidermal cells (Fig. 6). Later, large vesicles developed which became filled with pus cells.

In some of the lesions coagulative necrosis was also evident. Figures 7 and 8 illustrate two portions of a lesion stained by the OTA method, showing both vesiculation and coagulative necrosis. Gross swelling and vacuolation transformed many of the cells into a reticulum consisting of vesicles enclosed by films of necrotic protoplasm; from these vesicles most of the cellular content disappeared (Fig. 7). On the other hand, some necrotic cells were not swollen and appeared to have retained their protein; in this portion of the lesion coagulative necrosis occurred (Fig. 8).

Both the OTA and tetrazo methods revealed conspicuous loss of protein from the vacuolated cells. However, the intact keratin of the

stratum corneum was strongly colored by the tetrazo method, although only faintly tinted by OTA (Figs. 9 and 10). In the region of vesiculation, loss of tannophilic protein from the swollen vacuolated cells was a prominent feature of degenerative ballooning in vaccinia (Figs. 9 and 11).

Protein Content of Inclusion Bodies

The inclusion bodies in the cytoplasm of the epidermal cells in vaccinial lesions were stained deep salmon red by the OTA method. Without oxidation by periodic acid, however, the inclusions were only faintly colored by diazotized o-dianisidine. After masking with formaldehyde, the coloration of the inclusion bodies by OTA was substantially diminished.

Inclusion bodies in the proliferating epidermal cells at the edge of a vaccinial lesion were stained by the OTA method (Figs. 2 and 12). In the margins of the lesions the bodies were usually spherical and discrete and occurred singly in each affected cell. It was evident that the inclusions were remarkably rich in tannophilic protein (Fig. 12). Some of the uppermost cells of the malpighian layer exhibited early swelling, the onset of vacuolation, and contained single inclusion bodies (Fig. 13). The cells adjoining regions of vacuolation and vesiculation were at a later stage of infection and contained multiple inclusions consisting of granular aggregates which were much richer in protein than the more faintly stained nuclei still visible in some of the cells (Fig. 14).

In sections stained by the tetrazo method the inclusion bodies were colored dark red. On treatment with ferric chloride after tanning, instead of the usual OTA technique, the bodies stained blue-black. These observations establish the existence of abundant protein in the inclusion bodies. With the Feulgen method for deoxyribonucleic acid, the inclusions could only be found with difficulty; but in 2 of the 3 lesions examined by this method, it was apparent that some of the bodies were Feulgen-positive.

Discussion

The protein in the macromolecular fabric of dead and dying cells suffers various fates: firstly, it may persist in the necrotic cells in a mummified condition; secondly, it may be disintegrated by autolysis; and thirdly, it may be consumed by phagocytes.

Corper,[1] by chemical estimation, established that the greater part of the protein in the spleen was retained during ischemic necrosis even long after the nuclei had disappeared. Dixon,[2] by cytochemical observations, demonstrated the persistence of protein in necrotic cells in infarcts of the brain, spleen and kidney. Sometimes, however, protein

rapidly disappeared from necrotic cells. Wells[4] noted that after cloudy swelling, autolysis could be extremely rapid, and he suggested that cloudy swelling was a consequence of osmotic absorption of water into injured cells. Dixon[2] and Dixon and McCullagh[5] supposed that such osmotic absorption of water in damaged cells was an essential prelude to autolysis of protein. If the cells were so rapidly killed that their osmotic systems were destroyed, then no water was absorbed, no autolysis occurred, and protein persisted. If, on the other hand, the cells were at first less severely damaged, then their osmotic systems remained intact; in this case water was absorbed into the injured cells, and autolysis with concomitant loss of protein could proceed. This sequence of events probably accounts for the loss of protein which may occur in ischemic damage to the brain[2] and also for the hydropic swelling, accompanied by almost total destruction of cytoplasmic protein, frequently encountered in the cells of the liver after injury by carbon tetrachloride.[5] In the latter circumstance some of the damaged cells were swollen and hydropic and almost devoid of protein, while others which had not suffered swelling showed no loss of protein.[5]

The observations on vaccinial lesions confirm the view that aqueous or hydropic swelling is a necessary prelude to autolysis of protein. The swollen epidermal cells ultimately lost nearly all their protein, showed ballooning degeneration,[6] and became dilated into spongiose saccules or vesicles traversed by strands of persistent protoplasm. Himmelweit[15] suggested that ballooning of cells injured by vaccinia was an osmotic phenomenon due to disintegration of cytoplasm. As with aqueous swelling in other kinds of cellular injury, the ballooning in vaccinia could occur only if the osmotic systems of the cells were still intact; water was then absorbed and extensive autolysis of protein could proceed (Figs. 6, 7, and 9 to 11). If, on the other hand, the cells were so rapidly killed that their osmotic capacity was destroyed, then aqueous swelling was precluded; the water necessary for autolysis was therefore not absorbed. In the absence of autolysis, substantial quantities of intracellular protein were retained. Thus rapid cellular death, accompanied by destruction of the osmotic properties of the cells, probably accounted for the retention of protein in those necrotic cells of vaccinial lesions which escaped hydropic degeneration (Fig. 8).

The cytoplasmic inclusion bodies in vaccinia were rich in tannophilic protein, which stained salmon red by the OTA method. The reactive groups stainable by OTA were effectively masked by pretreatment with formaldehyde.[7] These observations indicated that the protein

of the inclusion bodies contained abundant basic $-NH_3+$ groups capable of binding tannic acid. The pronounced eosinophilia of the inclusions probably also depended on positively charged basic groups in the protein contained within them. The protein of the bodies could also be detected, after tanning, by treatment with ferric chloride.[11] In this case the inclusion bodies became blue-black in color. The presence of tyrosine, tryptophan and histidine groups in the protein accounted for their intense coloration by the tetrazo method.

According to Bland and Robinow,[16] vaccinia inclusions are Feulgen-positive at an early stage in their development, but eventually become Feulgen-negative. In 2 of the 3 epidermal lesions examined by the Feulgen method, positive inclusions were detected. The inclusions thus probably contained some deoxyribonucleic acid (DNA); this is consonant with the current belief that the inclusions are sites of maturation or multiplication of the virus. Some of the abundant protein in the bodies may therefore be viral nucleoprotein. But much of this protein is probably located in the matrix which is known to surround the viral particles in vaccinial inclusions.[15]

The inclusion bodies appeared to assimilate into themselves most of the cytoplasmic protein in the epidermal cells (Figs. 2, 12, and 14). We are ignorant of why infection by vaccinia causes ballooning, or indeed why the virus kills the epidermal cells at all. It is possible that the synthesis of protein in the inclusion bodies deflects some essential synthetic materials which are normally used for the manufacture of enzymic protein in the cell. Deprived of these materials, necessary for the manufacture of cellular protein, the cell may be injured irreversibly. Interference with synthetic processes dependent upon specialized enzymes, such as the formation of adenosine triphosphate, may result in catabolism of macromolecules, a rise in concentration of crystalloids, and an increase in osmotic pressure. Provided the osmotic systems of the cell are still intact, a rise in concentration of crystalloids would be followed by aqueous swelling and subsequent autolysis of protein. The final disintegration of the protein in the inclusion bodies of the ballooned cells may insure dissolution of the matrix and liberation of free elementary bodies; thus the viral particles may be disseminated after disruption of the host epithelium.

Summary

The content of protein in vaccinial lesions of the skin in young rabbits was studied by the oxidized tannin-azo (OTA) method.

Epidermis injured by vaccinia may either suffer aqueous swelling

with profound loss of protein, or show coagulative necrosis and retain its protein. Aqueous swelling with ballooning of the cells appears to be an essential prelude to autolysis.

The cytoplasmic inclusion bodies in the epidermal cells contain abundant tannophilic protein stainable by the OTA method and also by the tetrazo method of Danielli.

REFERENCES

1. Corper, H. J. Correlation of the histological and chemical changes in the spleen during necrosis and autolysis. *J. Exper. Med.*, 1912, **15**, 429–449.
2. Dixon, K. C. Persistence of protein in infarcts. *J. Path. & Bact.*, 1956, **71**, 37–43.
3. Dixon, K. C. Protein and nucleic acid in caseous necrosis. *Am. Rev. Tuberc.*, 1958, **77**, 106–119.
4. Wells, H. G. Chemical Pathology. W. B. Saunders Co., Philadelphia and London, 1925, p. 443.
5. Dixon, K. C., and McCullagh, G. P. Protein in dying liver cells. *Quart. J. Exper. Physiol.*, 1957, **42**, 104–112.
6. Unna, P. G. Die Histopathologie der Hautkrankheiten. In: Lehrbuch der speciellen pathologischen Anatomie. Ergänzungsband. II Thiel. Orth, J. (ed.). August Hirschwald, Berlin, 1894, pp. 445–447.
7. Dixon, K. C. Oxidized tannin-azo method for protein in tissues. *Am. J. Path.*, 1959, **35**, 199–211.
8. Danielli, J. F. A study of the techniques for the cytochemical demonstration of nucleic acids and some components of proteins. Symposia of the Society for Experimental Biology, University Press, Cambridge, England, 1947, Vol. I, pp. 101–113.
9. Dixon, K. C. Glycolysis and cytochemistry of cerebral cortex. *J. Physiol.*, 1953, **120**, 267–277.
10. Salazar, A. L. Méthode pour la coloration des éléments tannophiles: tannin-osmium; tannin-osmium-fer. *Compt. rend. Soc. de biol.*, 1921, **84**, 991–994.
11. Dixon, K. C. Differentiation of chromophilic and chromophobic neurones. *J. Anat.*, 1958, **92**, 425–432.
12. Tyzzer, E. E. The etiology and pathology of vaccinia. *J. Med. Research*, 1904, **11**, 180–229.
13. Hammerschmidt, J. Die Genese der Einschlusskörper in der Haut bei einigen Chlamydozoenerkrankungen. *Wien. klin. Wchnschr.*, 1918, **31**, 271.
14. Amies, C. R. The production of homogeneous suspensions of vaccinia elementary bodies and the histology of the associated skin lesions. *J. Path. & Bact.*, 1938, **47**, 205–222.
15. Himmelweit, F. Observations on living vaccinia and ectromelia viruses by high power microscopy. *Brit. J. Exper. Path.*, 1938, **19**, 108–123.
16. Bland, J. O. W., and Robinow, C. F. The inclusion bodies of vaccinia and their relationship to the elementary bodies studied in cultures of the rabbit's cornea. *J. Path. & Bact.*, 1939, **48**, 381–403.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Except when indicated differently, all illustrations are prepared from sections stained by the oxidized tannin azo (OTA) method.

FIG. 1. Normal skin of a young rabbit. The malpighian layer of epidermis and medullary cells of hair are intensely colored. The *stratum corneum* is very faintly stained. × 450.

FIG. 2. Zone of proliferation at the margin of a vaccinial lesion. Intensely colored inclusion bodies are visible in cells in the right half of the photograph. × 450.

FIG. 3. The same vaccinial lesion shown in Figure 2. The transition from normal skin through the zone of proliferation to the region of ballooning degeneration is evident. Hematoxylin and eosin stain. × 110.

FIG. 4. Higher magnification of portion of the field shown in Figure 3. The cells of the upper part of the malpighian layer are grossly swollen and vacuolated; eosinophilic inclusions as well as nuclei are present in the two topmost cells on the left. The cells in the lower part of the illustration are filled by eosinophilic inclusions. Hematoxylin and eosin stain. × 420.

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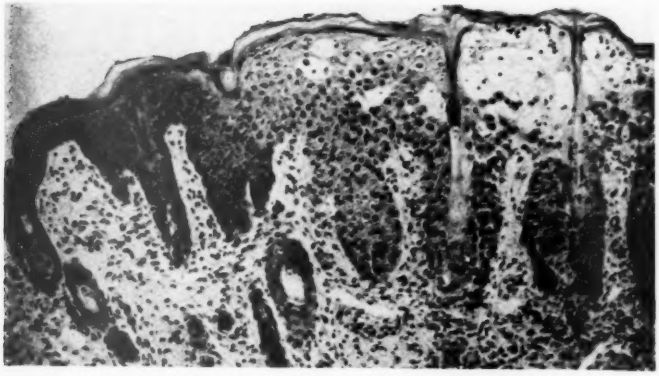

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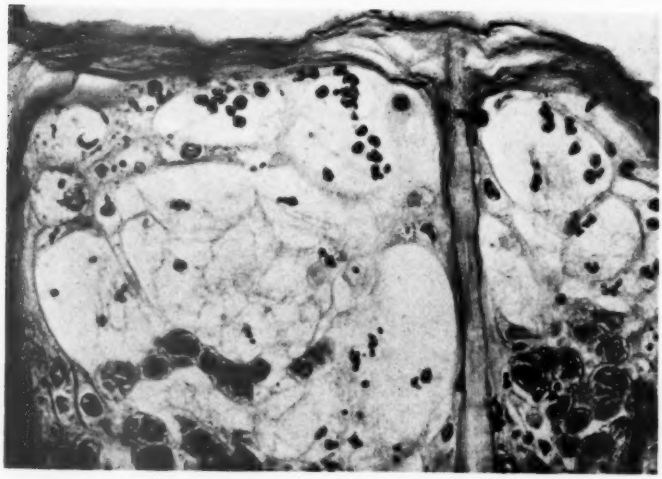

FIG. 5. Vaccinial lesion. This illustrates a central part of lesion shown in Figure 2. Some of the cells of the malpighian layer exhibit swelling and loss of protein. × 450.

FIG. 6. A later stage of development of the same lesion in Figures 2 and 5. Cells of the malpighian layer show vacuolation and gross loss of protein. × 420.

FIG. 7. Vaccinial lesion exhibiting intense swelling of cells in the upper malpighian layer with the formation of vesicles containing pus. There is gross loss of cellular protein. × 450.

FIG. 8. Another portion of the lesion shown in Figure 7. Necrotic cells in the upper malpighian layer exhibit little swelling and retain substantial quantities of protein. The *stratum corneum*, which is faintly stained, is visible at the upper margin of the photograph. × 450.

FIG. 9. A papular vaccinial lesion in which the central zone of ballooning degeneration is prominent. Protein has been lost from the swollen vacuolated cells. × 110.

FIG. 10. The papular lesion shown in Figure 9, stained by the tetrazo method. Loss of protein from the vacuolated cells is evident. × 110.

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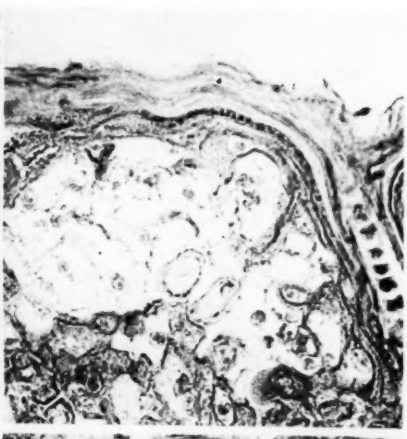

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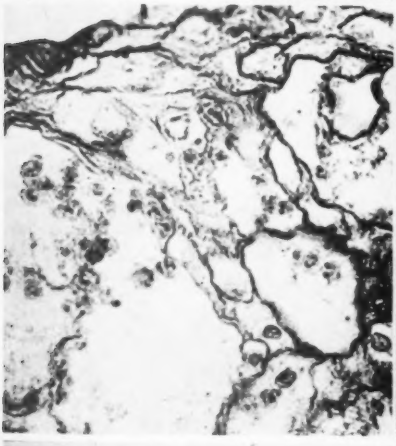

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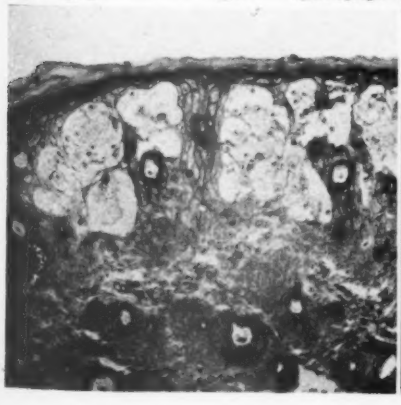

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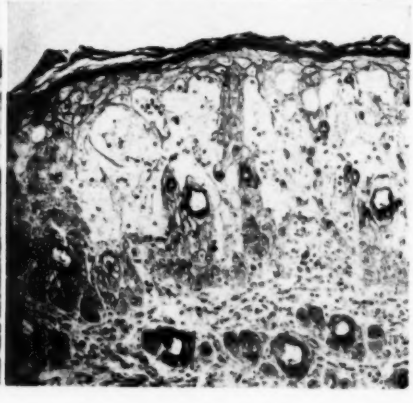

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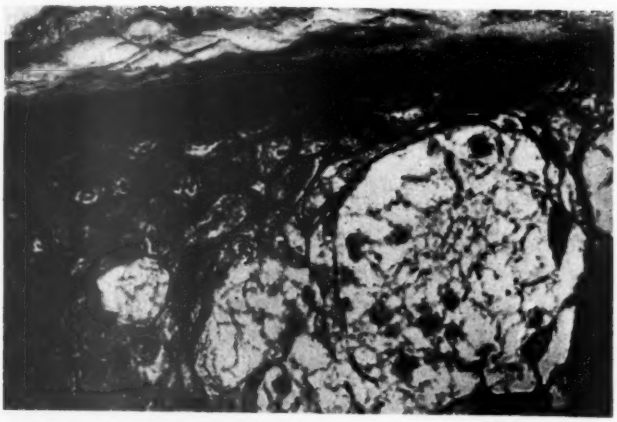
FIG. 11. Higher magnification of a portion of the lesion shown in Figure 9. The cells of the malpighian layer are intensely colored except for those which are vacuolated; the latter appear to have lost most of their cytoplasmic protein. × 450.

FIG. 12. Zone of proliferation in a vaccinial lesion. Discrete, spheroidal inclusion bodies are prominent in the cytoplasm of cells in the malpighian layer. These inclusions are much richer in tannophilic protein than the nuclei. × 1,150.

FIG. 13. Uppermost cells of the malpighian layer in a vaccinial lesion. Vacuolation is commencing in the cytoplasm. A single inclusion body, which is much richer in protein than the nucleus, is clearly visible in one of the cells. × 1,150.

FIG. 14. The central portion of the lesion shown in Figures 12 and 13. Some of the cells exhibit vacuolation; others are filled with irregular masses of inclusion material containing abundant tannophilic protein. Nuclei stain faintly, but are clearly visible in two of the cells. × 1,150.



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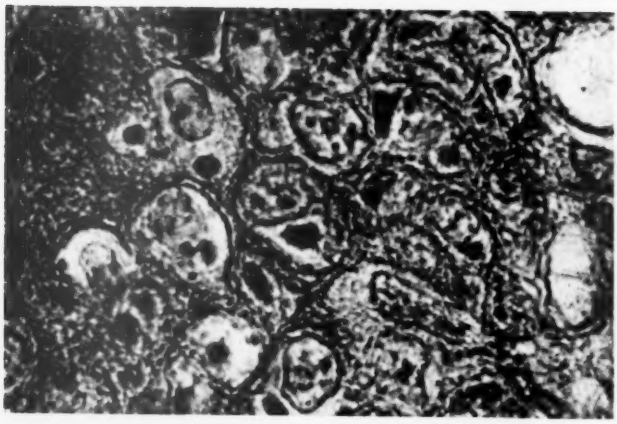

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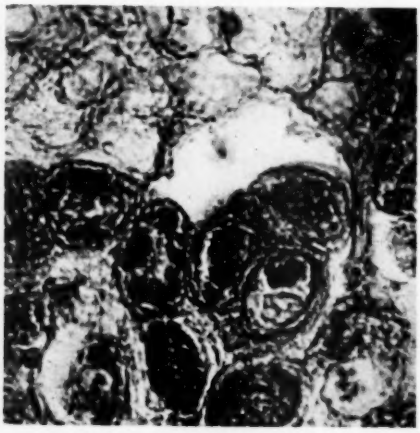

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THE MECHANISM OF FIBROSIS IN ARTERIOSCLEROSIS*

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In a recent publication[1] we reported two cases with multiple mural thrombi of the aorta, which showed all stages of organization from recent thrombus formation to complete incorporation of the lesion into the vascular wall as a fibrous plaque. The question whether or not all fibrous plaques arise from mural thrombi was left open.

The purpose of the study here reported was to search for evidence of old and recent thrombosis by examining a large number of fibrous plaques. Direct evidence in support of the concept that mural thrombosis is a common factor in the fibrosis of the intima was found in some plaques, and indirect evidence in others.

Material and Methods

Our studies were carried out almost exclusively on aortas, but in a few instances sections of renal, mesenteric and subclavian arteries were also examined.

Tissue from 148 cases was examined in our studies of arteriosclerosis. Fibrous plaques were procured from 65, a total of 140 being examined microscopically. Grossly, the lesions appeared as white-opaque, glassy-white, or pearly-white plaques, occasionally exhibiting a red or reddish-brown tinge. Processing of the tissue was carried out as described elsewhere.[2]

Results

Plaques which appeared white and opaque, or glassy-white grossly were made up almost entirely of recently formed thrombi. These were usually superimposed on partly or completely organized thrombi (Fig. 1). The recent thrombus was composed mainly of fibrin which had enmeshed numerous blood cells and platelets (Fig. 2). In the partly organized thrombus, the unorganized portion had a fibrinoid appearance, being intensely acidophilic, homogeneous, and refractile. The region undergoing early organization was composed of elongated cells and capillaries, surrounded by delicate reticulum fibrils and

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ground substance rich in acid mucopolysaccharide (AMP). With trichrome and phosphotungstic acid hematoxylin (PTAH) stains, not all of the recent thrombus stained in the manner of fibrin. That which did appeared intensely red with trichrome or dark blue with PTAH stains; other portions stained reddish-blue and light brown respectively (Fig. 3). Mural thrombi were occasionally large (Fig. 1), but often only a small, superficial "encrustation" could be seen (Fig. 4).

Pearly white plaques were frequently composed entirely of young AMP-rich connective tissue (Fig. 5).[3] The nature and the origin of the plaques from thrombi was suggested by the recognition of lesions of identical composition, which contained small remnants of unorganized thrombus (Fig. 6). Atheromas were frequently covered by fibrous caps and were grossly identical to pearly white plaques. The plaques overlying atheromas were also frequently composed of AMP-rich connective tissue and contained varying amounts of unorganized thrombus (Fig. 7).

The organization of the thrombus usually differed from that ordinarily encountered; it was not of the nature of granulation tissue. The cells proliferating in the AMP-rich ground substance were primitive or more mature smooth muscle cells. These, as a rule, had a longitudinal arrangement along the axis of the vessel. Occasionally there was disorientation, but the cells could still be recognized as smooth muscle elements, with elongated, somewhat wavy nuclei, elongated tapering cytoplasm and intensely acidophilic myofibrils (Fig. 8). Also present in the organizing thrombus at times were capillaries, which varied in size (Fig. 9). These, as in the case of the smooth muscle cells, were surrounded by delicate, periodic acid-Schiff (PAS)-positive basement membranes. Surrounding the capillaries, but also in the ground substance between smooth muscle cells, delicate collagen fibers had formed. In pentachrome II stained sections,[4] these collagen fibers stained red, contrasting with the blue ground substance. They could be demonstrated well, and distinguished from the PAS-positive basement membranes by Lillie's allochrome[5] stain, with which they stained intensely blue. The final outcome of the organizing process, whether occurring in plaques (Fig. 10) or in the caps covering atheromas (Fig. 7), was the formation of collagenous tissue, often with hyaline appearance. The smooth muscle in such lesions showed varying degrees of atrophy, resulting in the formation of clear, elongated spaces.

Lipids were occasionally present in lipophages in the organizing thrombi (Fig. 11), as well as in the caps covering atheromas (Fig.

12). At times, old collagenous plaques also contained numerous foam cells.

Direct evidence of the thrombotic origin of fibrous plaques was manifest in our material in the form of unorganized thrombotic remnants in 62 of 140 plaques. Organization similar to that seen in these plaques was also present in the remaining 78 plaques, although here no recognizable remnants of thrombi were detected. The stage of organization varied. The majority of the plaques were composed mainly of AMP-rich tissue; in others, collagen was more abundant. Some plaques, although containing abundant collagen, still exhibited remnants of unorganized thrombi.

Discussion

Because arteriosclerosis is so polymorphous, many investigators have considered it to represent not one, but two or even more different conditions, developing concomitantly but independently of each other. One form characteristic of arteriosclerosis is the white plaque. White plaques vary in size and shape and are usually oriented with the long axis of the vessel. They were found either unassociated with an atheroma, or in the form of white caps covering the latter. The derivation of these focal lesions from blood was well recognized by Rokitansky over 100 years ago. According to him, the fibrinous substance of the blood was deposited on the surface of the arterial wall and later through fatty metamorphosis became an atheroma. He stated: "The deposit is an endogenous product derived from the blood, and for the most part from the fibrin of the arterial blood. Its formation demonstrates the pre-existence of a peculiar crasis of the blood, which is intrinsically arterial."[6] In subsequent years, however, the white plaques were thought to represent reactive fibrosis secondary to primary fat deposition within the substance of the intima. Only recently Clark, Graef and Chasis,[7] and Duguid[8-11] have re-examined the white plaque and have reconsidered its origin from the blood. They, as well as Crawford and Levene,[12] believed that disturbances in the clotting mechanism were responsible for the formation of the precursor thrombi. Harrison,[13] by giving repeated intravenous injections of broken up blood clot, produced a similar type of arteriosclerosis of the pulmonary arteries in healthy rabbits.

We believe fully with Rokitansky, as well as with the British investigators, that the white plaque takes its origin from blood constituents. The usual manner in which this process begins is by the formation of thrombi.[14] On the other hand, the white plaque may also be the out-

come of an "insudation," as mentioned in previous publications.[15,16] We have found that a plaque was often composed of several layers, each representing a thrombus of a different age. We have not encountered any fibrosis in our material which could have resulted as a reaction to the deposition of lipids in the intima.

We have observed, as did Crawford and Levene,[12] that the organization of thrombi in the aortic wall was different from the process of organization commonly encountered elsewhere. The aortic (arteriosclerogenic) thrombi were predominantly organized by means of an avascular process. The cells concerned with this were smooth muscle cells. It is remarkable that we were only occasionally able to find fibroblasts in the organizing thrombi.

Summary

These studies have revealed that the common white plaque in arteriosclerosis, whether or not accompanied by atheroma, arises by means of organization of thrombi. Occasional remnants of thrombus were seen in organizing fibrous plaques. In other plaques the pattern of the fibrosis was so like that noted in plaques arising from mural thrombi that it seemed most reasonable to conclude that the former, too, originated in this fashion.

A peculiar feature of the organization of thrombi in this location was the constant participation of cells possessing all the morphologic features of smooth muscle cells.

REFERENCES

1. More, R. H.; Movat, H. Z., and Haust, M. D. Role of mural fibrin thrombi of the aorta in genesis of arteriosclerotic plaques. Report of two cases. *A. M. A. Arch. Path.*, 1957, **63**, 612–620.
2. Movat, H. Z., and More, R. H. The nature and origin of fibrinoid. *Am. J. Clin. Path.*, 1957, **28**, 331–353.
3. Bunting, C. H., and Bunting, H. Acid mucopolysaccharides of the aorta. *A. M. A. Arch. Path.*, 1953, **55**, 257–264.
4. Movat, H. Z. Demonstration of all connective tissue elements in a single section. *A. M. A. Arch. Path.*, 1955, **60**, 289–295.
5. Lillie, R. D. Histopathologic Technic and Practical Histochemistry. The Blakiston Co., New York and Toronto, 1954, ed. 2, 501 pp.
6. Rokitansky, C. A Manual of Pathological Anatomy. Day, G. E. (transl.). The Sydenham Society, London, 1852, Vol. IV, p. 272 Cited by Duguid, 1949[9].
7. Clark, E.; Graef, I., and Chasis, H. Thrombosis of the aorta and coronary arteries, with special reference to the "fibrinoid" lesions. *Arch. Path.*, 1936, **22**, 183–212.
8. Duguid, J. B. Thrombosis as a factor in the pathogenesis of aortic atherosclerosis. *J. Path. & Bact.*, 1948., **60**, 57–61.
9. Duguid, J. B. Pathogenesis of arteriosclerosis. *Lancet*, 1949, **2**, 925–927.

10. Duguid, J. B. The arterial lining. *Lancet*, 1952, **2**, 207–208.
11. Duguid, J. B., and Anderson, G. S. The pathogenesis of hyaline arteriosclerosis. *J. Path. & Bact.*, 1952, **64**, 519–522.
12. Crawford, T., and Levene, C. I. The incorporation of fibrin in the aortic intima. *J. Path. & Bact.*, 1952, **64**, 523–528.
13. Harrison, C. V. Experimental pulmonary arteriosclerosis. *J. Path. & Bact.*, 1952, **60**, 289–293.
14. Haust, M. D.; Movat, H. Z., and More, R. H. The role of fibrin thrombi in the genesis of the common white plaque in arteriosclerosis. (Abstract) *Circulation*, 1956, **14**, 483.
15. Movat, H. Z., and More, R. H. Morphologic and histochemical studies on the development and progression of arteriosclerosis. (Abstract) *Circulation*, 1955, **12**, 484.
16. Movat, H. Z.; Haust, M. D., and More, R. H. The morphologic elements in the early lesions of arteriosclerosis. *Am. J. Path.*, 1959, **35**, 93–101.

[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. A recent thrombus (A) is seen superimposed upon an older one (B). Within the substance of the intima, remnants of fibrinous material (C) are seen in coalescent form; this represents a portion of a still older, partly organized thrombus. Phosphotungstic acid hematein stain. × 28.6.

FIG. 2. Higher power view of Figure 1 (A). The recent thrombus is made up of fibrin in which numerous cells and platelets are enmeshed. Phosphotungstic acid hematein stain. × 190.

FIG. 3. A recent thrombus (A) is seen superimposed on a partly organized thrombus (B). The unorganized portions (black in the photograph, orange in the section) of the latter have a fibrinoid appearance. Similar material is seen deeper (C), as part of a still older thrombus. Alcian blue, periodic acid-Schiff, hematoxylin, and orange G stains. × 81.6.

FIG. 4. Superficial "encrustation." Coalescent fibrinous material is seen as black bands (dark blue in the section). Phosphotungstic acid hematein stain. × 190.

FIG. 5. The pearly white plaque shown in this photograph is composed entirely of young connective tissue rich in acid mucopolysaccharide (light gray in the photograph, light blue in the section). Numerous elongated cells (stained red in the section) are mainly seen in the upper part of the plaque. Heidenhain's azan stain. × 28.6.

FIG. 6. Small remnants of unorganized thrombus (A) (red staining in the section, black in the photograph) betray the nature of this lesion. They represent part of a large thrombus, which has been almost completely organized. The organized portions consist of the homogeneous light gray material above and below the black clumps (A). Hemalum, phloxine and saffron stains. × 184.

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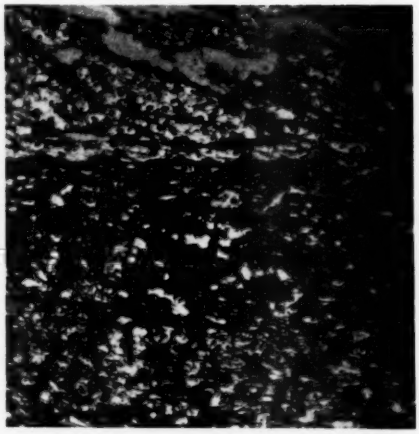

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FIG. 7. Fibrous cap (A) covering an atheroma (B) is made up of AMP-rich connective tissue, hyalinized at the base. It represents an organized thrombus which still contains unorganized portions (C). The latter is good evidence that at an earlier date the whole fibrous cap had an appearance similar to the thrombus seen in Figures 1 and 3. Hemalum, phloxine and saffron stains. × 28.6.

FIG. 8. Higher power view of the organizing thrombus seen in Figure 7 shows numerous cells proliferating in the AMP-rich ground substance. The cells contain delicate fibrils on either side of their nuclei. Hemalum, phloxine and saffron stains. × 213.

FIG. 9. Capillaries present in the organizing thrombus. The unorganized fibrinous material is seen as dark gray homogeneous bands (orange in the section) (B). The ground substance (blue staining in the section) is the light gray homogeneous material. The basement membrane of the capillary (PAS-positive, i.e., purple) is seen in black (A). Alcian blue, PAS, hematoxylin and orange G stains. × 410.

FIG. 10. Higher power view of a thrombus completely converted into a collagenous plaque, containing atrophic smooth muscle cells. The black bands (blue stained in the section) represent collagenization and hyalinization of the structures surrounding capillaries and smooth muscle cells. Masson's trichrome stain. × 80.

FIG. 11. Lipophages (A) present in an organizing thrombus (B). Carbowax section; Fettrot and hematoxylin stains. × 80.

FIG. 12. Organizing thrombus (A) covering an atheroma (B), and containing numerous lipophages (C). Lipophages are seen also within the atheroma. Carbowax section; Fettrot and hematoxylin stains. × 28.6.

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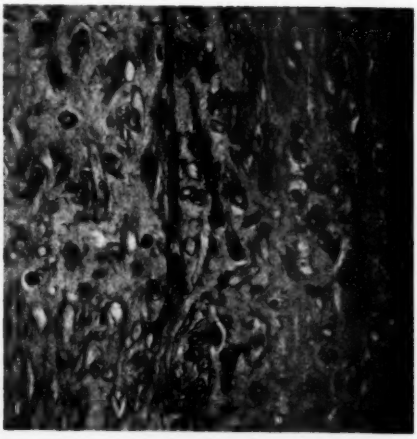

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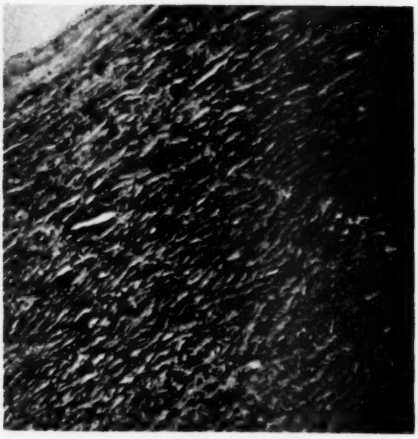

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LOCALIZATION OF COLLOIDAL SUBSTANCES IN VASCULAR ENDOTHELIUM. A MECHANISM OF TISSUE DAMAGE

II. Experimental Serum Sickness with Acute Glomerulonephritis Induced Passively in Mice by Antigen-Antibody Complexes in Antigen Excess *

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Rich and Gregory produced experimental serum sickness in rabbits by injections of large amounts of horse serum.[1] The characteristic lesions described were proliferative glomerulonephritis, necrotizing arteritis and endocarditis.[2-4] The possible relationships of these pathologic alterations to human disease have been thoroughly discussed.[3-5]

Since the original description, the immunologic mechanisms associated with the occurrence of these lesions in rabbits have been extensively investigated. Hawn and Janeway demonstrated that the same pathologic pattern could be produced by the injection of large amounts of purified serum proteins.[6] Germuth showed, by careful analysis of the immunologic events as related to the histologic alterations, that the lesions developed during the immune phase of antigen elimination and regressed when free antibody appeared in the circulation.[7] The immune phase of antigen elimination was of short duration and was associated with the presence of soluble antigen-antibody complexes in the circulation.[8,9] Dixon, Vazquez, Weigele and Cochrane,[9] using the fluorescent labeled antibody technique, showed the presence of antigen and antibody in the glomerular and vascular lesions of serum sickness.

It has been postulated, therefore, that the local presence of antigen and antibody brings about the lesions. It appears unlikely that their localization results from the reaction of antibody with fixed antigen, inasmuch as the lesions first appear at a time when excess of antigen exists in the circulation, and antibody, as it is produced, is immediately bound in the form of antigen-antibody complexes. Furthermore, Dixon and his associates failed to demonstrate the presence of antigen in glomeruli, heart valves or arteries prior to the appearance of lesions.[9] It seems reasonable, therefore, to assume that soluble antigen-antibody

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complexes are localized as such in glomeruli, arteries and endocardium, and are responsible for the lesions.

In the present study, experimental serum sickness was produced passively in normal mice within a 36-hour period by the intravenous injection of soluble antigen-antibody complexes. In addition, observations are reported on the production of mild acute glomerulonephritis by the administration of histamine and human gamma globulin.

Material and Methods

The experiments were performed on male white Swiss Webster mice, weighing from 20 to 30 gm. Mice were used because their size allowed the use of smaller amounts of antigen-antibody complexes than required for rabbits.

Antiserums against 3 times recrystallized chicken ovalbumin were produced in a group of 5 rabbits, and in another group of 5 rabbits, against bovine serum albumin (BSA; Armour). The rabbits were immunized as follows: 10 mg. of antigen in complete Difco adjuvant were injected subcutaneously; two weeks later the animals received intravenous injections of alum-precipitated antigen; 3 doses of 3 mg. were given during the first week and 3 doses of 6 mg. during the second week. The rabbits were bled one week after the last injection. The serums with highest titers in each group were pooled and analyzed for antibody by the quantitative precipitin technique.[10] The antiovalbumin pool contained 4.6 mg. of antibody protein per ml. The anti-BSA pool contained 2.3 mg. of antibody protein per ml.

The mice were sacrificed by decapitation, and sections were prepared of heart, lung, liver, stomach, spleen, skin and kidney.

Experimental Procedures

The Production of Serum Sickness by Soluble Antigen-Antibody Complexes

In the first experiment, 2 groups of mice (IA and IB) received daily injections of rabbit antiserum containing excess antigen, as described below, for 5 days. The mice were sacrificed on the sixth day. For the first group (IA), 20 mg. of ovalbumin in 2 ml. of saline were added to 10 ml. of antiovalbumin serum containing 46 mg. of antibody. This resulted in slight precipitation. An injection of 1.2 ml. of this mixture, which contained 4.6 mg. of antibody and 2 mg. of ovalbumin, was administered intravenously to each of 8 mice. All of the mice showed some signs of anaphylactic shock, and 3 of the mice died within 2 hours. The other 5 mice recovered and were given the same dose of antigen-antibody mixture daily for 5 days. After the first

injection, the animals showed only minimal or no signs of anaphylaxis. For the second group (IB), 20 mg. of BSA in 2 ml. of saline were added to 10 ml. of anti-BSA serum which contained 23 mg. of antibody. There was no precipitation. Each of 6 mice received intravenous injections of 1.2 ml. of this mixture, which contained 2.3 mg. of antibody and 2 mg. of antigen. All of the mice survived. The injections were repeated daily for 5 days.

In the second experiment the same antiserum antigen mixtures were injected every 12 hours for 3 injections into 2 groups of 6 animals (IIA, ovalbumin; and IIB, BSA). Three animals from each group were sacrificed a few hours later and the others 24 hours later.

As controls to experiments I and II, 2 groups of 8 mice received injections of mixtures of normal nonimmune rabbit serum with either ovalbumin or BSA added, using the same amounts and schedules as in the experimental groups.

In the third experiment, 2 groups of 5 mice, IIIA and IIIB, were given injections with redissolved antigen-antibody complexes prepared as follows: For the first group (IIIA), the antibody in 3 ml. of anti-ovalbumin serum, which contained 13.8 mg. of antibody, was precipitated at its equivalence point. After repeated washing with saline, the precipitate was redissolved in 2 ml. of saline containing 50 mg. of ovalbumin. Twenty minutes later, this had dissolved and was injected into 5 mice, each receiving 2.76 mg. of antibody in the form of soluble antigen-antibody complexes. This was repeated every 12 hours for 3 injections. For the second group (IIIB), the antibody in 5 ml. of anti-BSA serum was precipitated at its equivalence point and washed repeatedly with saline. The precipitate, which contained 11.5 mg. of antibody, was redissolved with 25 mg. of BSA in 2 ml. of saline. Each of 5 mice was given 2.3 mg. of anti-BSA in the form of soluble antigen-antibody complexes. This was repeated every 12 hours for 3 injections. All mice in group IIIA and IIIB were sacrificed a few hours after the last injection.

Pathologic Observations. The pathologic features in all of the experimental groups were similar and will be described together. Minor differences between the groups will be pointed out. None of the 15 control mice showed any of the pathologic alterations to be described.

All of the 33 mice which survived to the end of the experiments showed acute glomerulonephritis. In the majority of cases the lesions were severe and affected all glomeruli. The glomeruli were enlarged and heavily infiltrated with neutrophils, many of which were fragmented (Figs. 2 to 4). The capillary lumens were narrowed and relatively bloodless. There was swelling of glomerular endothelial and

epithelial cells. A rare glomerulus showed partial necrosis. No alteration of the basement membranes was seen in sections stained with hematoxylin and eosin or periodic acid-Schiff (PAS) stains. In a few cases, the amount of neutrophil infiltration was not very marked, and the major changes were swelling and proliferation of glomerular cells with reduction in size of the capillary lumens (Figs. 5 and 6).

In group IIA, which received antiovalbumin serum with excess antigen every 12 hours for 3 injections, an additional feature was present in the glomeruli of 3 animals. There was accumulation of homogeneous, intensely eosinophilic, strongly PAS-positive material within capillary lumens (Fig. 7). This probably represented precipitated antigen-antibody complex. The inflammatory reaction in these glomeruli was milder than in most of the other animals. The most uniformly severe glomerular lesions were found in group IIIA which received redissolved precipitates of rabbit antiovalbumin.

In 10 of the 33 mice, acute endocarditis also was found. Instances of this were seen in each valve, but the mitral valve was most severely affected, especially near its point of attachment. The valve leaflet was thickened and infiltrated with neutrophils, mononuclear cells and Anitschkow myocytes (Fig. 8). In 3 cases there were small areas of coagulative necrosis in the myocardium.

Necrotizing arteritis was found in 4 of the 33 mice; in 3 instances in the heart, and in 1 in the stomach. The vessels involved were small muscular arteries, and in 3 animals they showed segmental necrosis of the media which was infiltrated by mononuclear cells and neutrophils. There were proliferation of endothelium and some perivascular inflammatory reaction (Fig. 9). In one instance there was extensive fibrinoid necrosis of the media of a coronary artery (Fig. 10). The number of cases in which arteritis or endocarditis was found may not represent the true incidence of these lesions in view of the fact that only one section was taken of each organ examined.

No granulomatous lesions were found in the spleen.

In another experiment (IV), each of 12 mice received 1.4 mg. of antiovalbumin with excess antigen every 48 hours for 3 doses. Similar lesions to those described were found in the kidneys, but they were mild and affected relatively few glomeruli.

The Production of Acute Glomerulonephritis by the Systemic Administration of Histamine and Human Gamma Globulin

The localization of antigen-antibody complexes in glomeruli and blood vessels leads to an inflammatory reaction through mechanisms

which are not understood, but which may involve complement. An attempt was made to produce similar glomerular lesions by the localization of human gamma globulin in glomeruli through the systemic administration of histamine. Histamine injection had been shown to bring about fixation of colloidal material in glomeruli.[11] Human gamma globulin was chosen because of the possibility that the fixation of antibody protein alone might be damaging to tissues.

The following experiment was performed: Three groups of 6 mice were treated as follows: Group V received 10 mg. of human gamma globulin (Squibb) intravenously in the form of a 2 per cent solution in saline, followed immediately by 10 mg. of histamine intraperitoneally. This was repeated daily for 5 days. Group VI received only 10 mg. of histamine daily for 5 days. Group VII received 10 mg. of human gamma globulin daily for 5 days. All mice were sacrificed on the sixth day. The animals of Group VI and VII, given histamine or globulin alone, showed no lesions in glomeruli or elsewhere. The animals in Group V had mild acute glomerulitis, which appeared in a minority of glomeruli (Fig. 11). These lesions were not nearly so severe or widespread as those seen in animals given antigen-antibody complexes. No arteritis was found in routine sections of any organ.

Discussion

The pathologic pattern of experimental serum sickness was produced in mice by the administration of soluble antigen-antibody complexes. To our knowledge, there have been no previous reports on the production of serum sickness in experimental animals except in the rabbit. The present observations indicate that, provided sufficient amounts of antigen and antibody are used, the morphologic alterations can be reproduced readily. The lesions were induced in normal nonsensitized animals by the passive administration of soluble antigen-antibody complexes. The rapidity with which the lesions appeared excludes the participation of an immune response by the mouse in the pathogenesis of the disorder. Similar conclusions were reached by Germuth and Pollack on the basis of experiments in which comparable lesions occurred within several days following the infusion of large amounts of antibody in rabbits previously receiving injections of antigen.[12] The lesions they described in the kidney were not so severe or so uniform as those observed in the present study.

The production of glomerulonephritis, arteritis and endocarditis by the passive administration of antigen-antibody complexes, in view of the demonstration of the presence of antigen and probably antibody at

the sites of tissue damage by Dixon and associates,[9] establishes the fact that the lesions are the result of the localization of antigen-antibody complexes as such.

It remains to be explained how these complexes are arrested in the tissues affected in serum sickness. Antigen-antibody complexes have been shown to behave in the circulation like unstable colloidal particles, in that they are readily phagocytized by the cells of the reticuloendothelial system.[13] As demonstrated in a previous study,[11] such colloidal particles can be localized in glomeruli and vascular endothelium in various organs under the influence of agents such as histamine, known to be released by antigen-antibody complexes.[14] For these reasons it is postulated that in serum sickness, antigen-antibody complexes, by releasing substances such as histamine, produce changes in endothelium of glomeruli and blood vessels. The alterations thus induced lead to the arrest of the complexes in these sites, and, in turn, to the tissue damage which ensues.

The glomerular lesions induced by antigen-antibody complexes in the present study were severe and widespread. They were characterized by a more intense exudative reaction than that observed in the lesions generally described in serum sickness in the rabbit. In this respect they more nearly resembled the lesions of acute glomerulonephritis in man. The results emphasize, as previously indicated by the work of Germuth and his associates,[7,15] and Dixon and co-workers,[9] that tissue damage based upon immunologic mechanisms need not involve an antibody directed against a component of the tissue, but may result from the mere localization in vascular endothelium of preformed antigen-antibody complexes. It should be mentioned that such a mechanism may be operative in the pathogenesis of human disease in which bacterial as well as drug antigens are involved.

Although in this study no attempt was made to determine precisely the minimal amount of antigen-antibody complexes required, relatively large amounts were needed to obtain uniformly severe lesions. Two different antigen-antibody systems were employed in the present experiments. The glomerulonephritis obtained with the antiovalbumin system was generally more severe than that with the anti-BSA system. The reason for this difference may be the somewhat larger amount used or the result of the more rapid excretion of ovalbumin[16] with consequent formation of progressively larger complexes in the circulation as the excess of antigen is reduced. This probably also occurs with the anti-BSA system but to a lesser extent.

In the course of these experiments, anaphylaxis was observed in

mice following the first injection of soluble antigen-antibody complexes, with death occurring in some cases with the higher doses. These observations, in conjunction with the recent report of Tokuda and Weiser,[17] extend to mice the observations of Germuth and McKinnon[14] on the production of anaphylaxis in the guinea pig by soluble antigen-antibody complexes.

It is interesting to compare the amounts of antibody which in the present study were shown to produce the lesions of serum sickness in mice with those estimated by Germuth, Flanagan and Montenegro[15] to be required for the induction of serum sickness in the rabbit. In the experiments in which severe lesions appeared after 3 injections of redissolved antigen-antibody precipitates during a 24-hour period, the total amount of antibody used was 8.28 mg. of antiovalbumin or 6.9 mg. of anti-BSA per mouse. The mice averaged 25 gm. in weight; this amounted to about 330 mg. of antiovalbumin per kg. or about 275 mg. of anti-BSA per kg. Germuth and his colleagues[15] estimated that the minimal amount of anti-BSA required to produce the lesions in the rabbit was about 40 mg. of antibody nitrogen for a 2 kg. rabbit, on the basis of the amount of antigen necessary. Converted to antibody protein per kg., this amounts to 125 mg. of anti-BSA per kg. In experiments in which these investigators infused antibody for a period of several days following the intravenous injection of BSA, larger amounts of anti-BSA were used. These varied from 119 mg. to 192 mg. of antibody nitrogen for a 2 kg. rabbit. In terms of antibody protein per kg., this ranged from 372 mg. to 600 mg. of anti-BSA per kg. It is interesting that the amounts of rabbit antibody used to produce the pathologic changes of serum sickness passively have been found to be of the same order of magnitude in mice and rabbits.

It is suggested that the mouse provides a convenient experimental animal in which to study the various mechanisms in the pathogenesis of these lesions. Studies are in progress to investigate further the quantitative aspects of antigen and antibody required for the development of acute lesions and to determine whether irreversible changes associated with evolution into a chronic form of the disease can be brought about by more prolonged treatment with antigen-antibody complexes.

Summary

The intravenous injection of soluble antigen-antibody complexes into mice, using rabbit antibody against ovalbumin or bovine serum albumin, produced the characteristic pathologic alterations of experimental serum sickness. Severe acute glomerulonephritis, necrotizing

arteritis, and acute endocarditis were observed within 36 hours after the first injection.

Evidence has been presented to show that the lesions were brought about by the localization of preformed antigen-antibody complexes at sites where tissue damage occurred. The mechanism responsible for this localization is discussed.

Passive anaphylaxis was observed in mice after injection of soluble antigen-antibody complexes.

REFERENCES

1. Rich, A. R., and Gregory, J. E. The experimental demonstration that periarteritis nodosa is a manifestation of hypersensitivity. *Bull. Johns Hopkins Hosp.*, 1943, **72**, 65–88.
2. Rich, A. R., and Gregory, J. E. Experimental evidence that lesions with the basic characteristics of rheumatic carditis can result from anaphylactic hypersensitivity. *Bull. Johns Hopkins Hosp.*, 1943, **73**, 239–264.
3. Rich, A. R., and Gregory, J. E. Further experimental cardiac lesions of the rheumatic type produced by anaphylactic hypersensitivity. *Bull. Johns Hopkins Hosp.*, 1944, **75**, 115–134.
4. Gregory, J. E., and Rich, A. R. The experimental production of anaphylactic pulmonary lesions with the basic characteristics of rheumatic pneumonitis. *Bull. Johns Hopkins Hosp.*, 1946, **78**, 1–12.
5. Rich, A. R. The pathology and pathogenesis of experimental anaphylactic glomerulonephritis in relation to human acute glomerulonephritis. *Bull. Johns Hopkins Hosp.*, 1956, **98**, 120–151.
6. Hawn, C. V., and Janeway, C. A. Histological and serological sequences in experimental hypersensitivity. *J. Exper. Med.*, 1947, **85**, 571–590.
7. Germuth, F. G., Jr. A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type. *J. Exper. Med.*, 1953, **97**, 257–282.
8. Dixon, F. J., and Maurer, P. H. Fate of antibody following *in vivo* combination with specific antigen. *Proc. Soc. Exper. Biol. & Med.*, 1953, **84**, 442–446.
9. Dixon, F. J.; Vazquez, T. T.; Weigle, W. O., and Cochrane, G. G. Pathogenesis of serum sickness. *A. M. A. Arch. Path.*, 1958, **65**, 18–28.
10. Kabat, E., and Meyer, M. M. Experimental Immunochemistry. Charles C Thomas Co., Springfield, Ill., 1948, 567 pp.
11. Benacerraf, B.; McCluskey, R., and Patras, D. Localization of colloidal substances in vascular endothelium. A mechanism of tissue damage. I. Factors causing the pathologic deposition of colloidal carbon. *Am. J. Path.*, 1959, **35**, 75–91.
12. Germuth, F. G., Jr., and Pollack, A. D. The production of lesions of serum sickness in normal animals by the passive transfer of antibody in the presence of antigen. *Bull. Johns Hopkins Hosp.*, 1958, **102**, 245–262.
13. Benacerraf, B.; Sebestyen, M., and Cooper, N. S. Phagocytosis of antigen antibody complexes by the reticuloendothelial system. *J. Immunol.* (in press).

14. Germuth, F. G., Jr., and McKinnon, G. E. Studies on the biological properties of antigen-antibody complexes. I. Anaphylactic shock induced by soluble antigen-antibody complexes in unsensitized normal guinea pigs. *Bull. Johns Hopkins Hosp.*, 1957, **101**, 13–44.

15. Germuth, F. G., Jr.; Flanagan, C., and Montenegro, M. R. The relationships between the chemical nature of the antigen, antigen dosage, rate of antibody synthesis and the occurrence of arteritis and glomerulonephritis in experimental hypersensitivity. *Bull. Johns Hopkins Hosp.*, 1957, **101**, 149–169.

16. Lapresle, C., and Grabar, P. Etude de l'influence de l'élimination rénale sur l'antigénicité des protéides. *Ann. Inst. Pasteur*, 1952, **83**, 705–712.

17. Tokuda, S., and Weiser, R. S. Production of anaphylaxis in the white mouse with soluble antigen-antibody complexes. *Science*, 1958, **127**, 1237–1238.

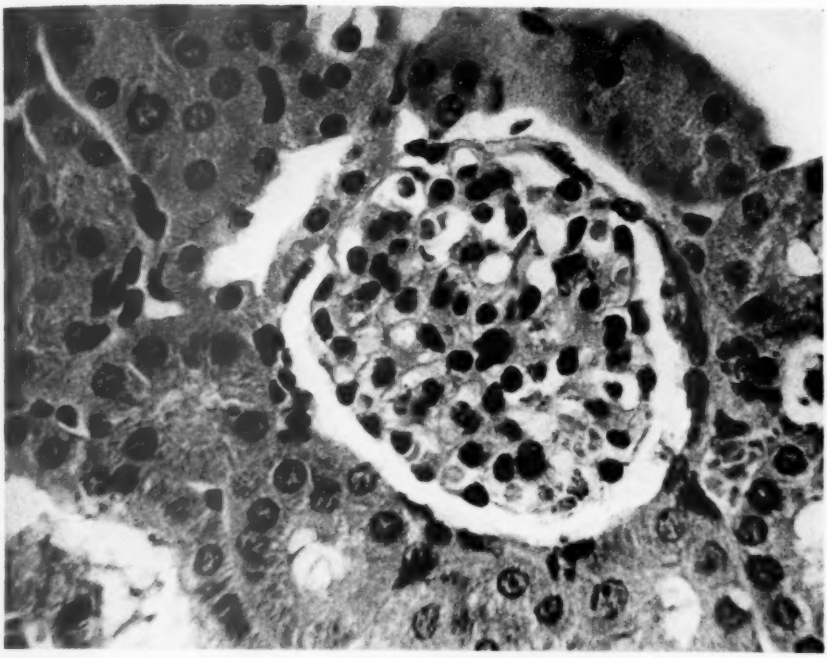
[*Illustrations follow*]

LEGENDS FOR FIGURES

Sections were stained with hematoxylin and eosin except where indicated.

FIG. 1. Normal mouse glomerulus. × 450.

FIG. 2. Glomerulus from mouse given 3 injections of redissolved antigen-antibody complexes in 24 hours. Mouse sacrificed 36 hours after the first injection (ovalbumin antiovalbumin, Experiment IIIA). There is swelling of glomerular cells and infiltration with neutrophils. The interstitial tissue and tubules are normal. × 450.



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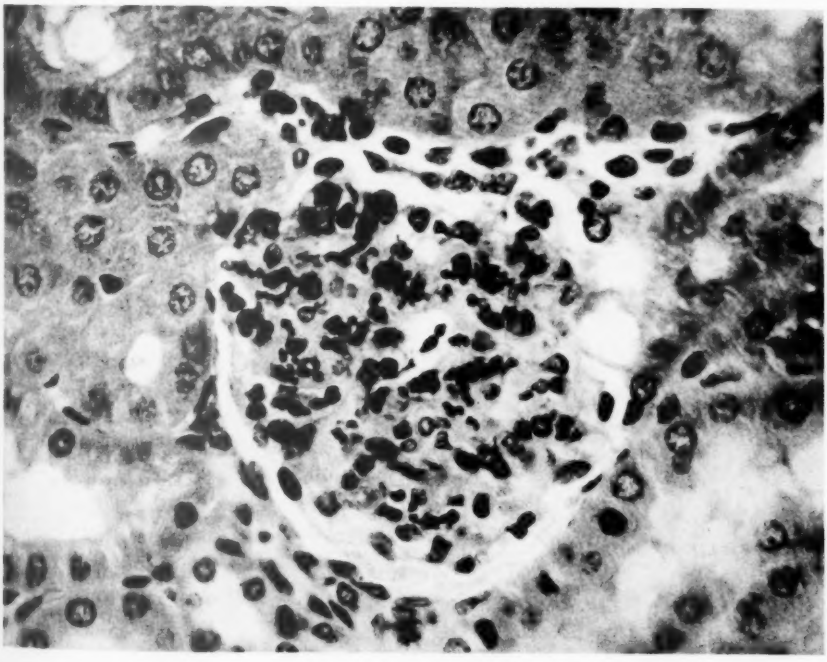

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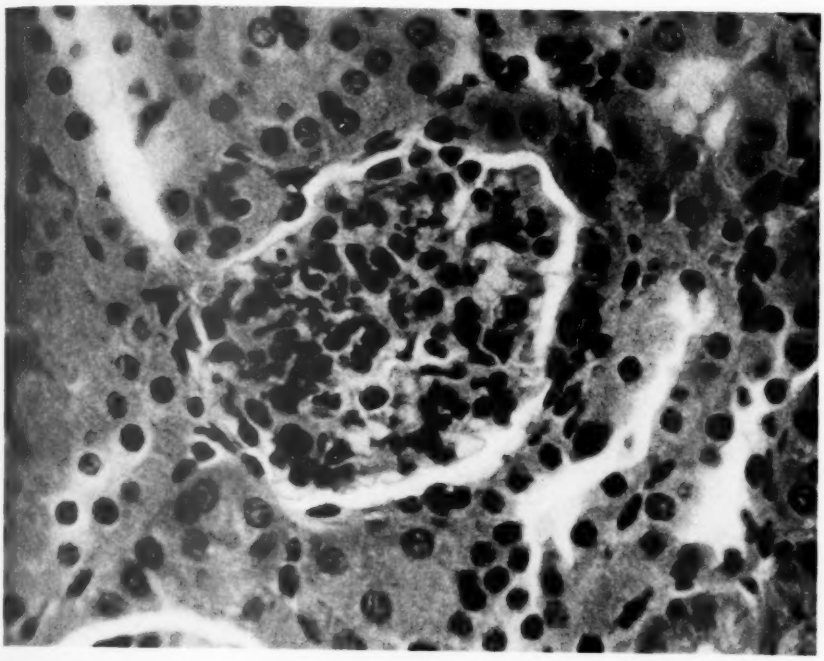
FIG. 3. Glomerulus from mouse given rabbit antiovalbumin serum with excess ovalbumin daily for 5 days (Experiment IA). The glomerulus is hypercellular, infiltrated with neutrophils and is relatively bloodless. × 450.

FIG. 4. Glomerulus from mouse given 3 injections of redissolved antigen-antibody complexes in 24 hours. Mouse sacrificed 36 hours after the first injection. (BSA-anti-BSA, Experiment IIIB). The glomerulus shows severe acute inflammatory reaction and hypercellularity. × 450.



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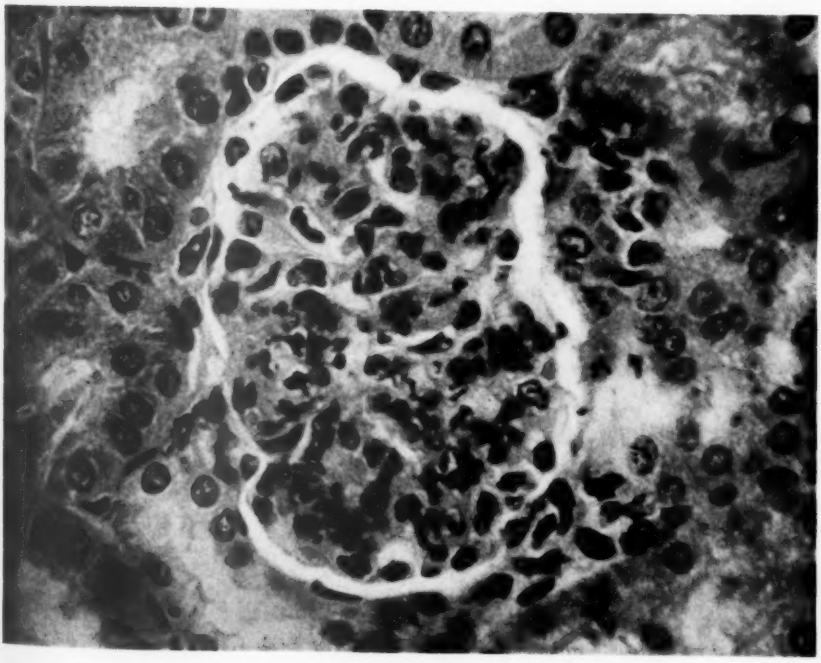

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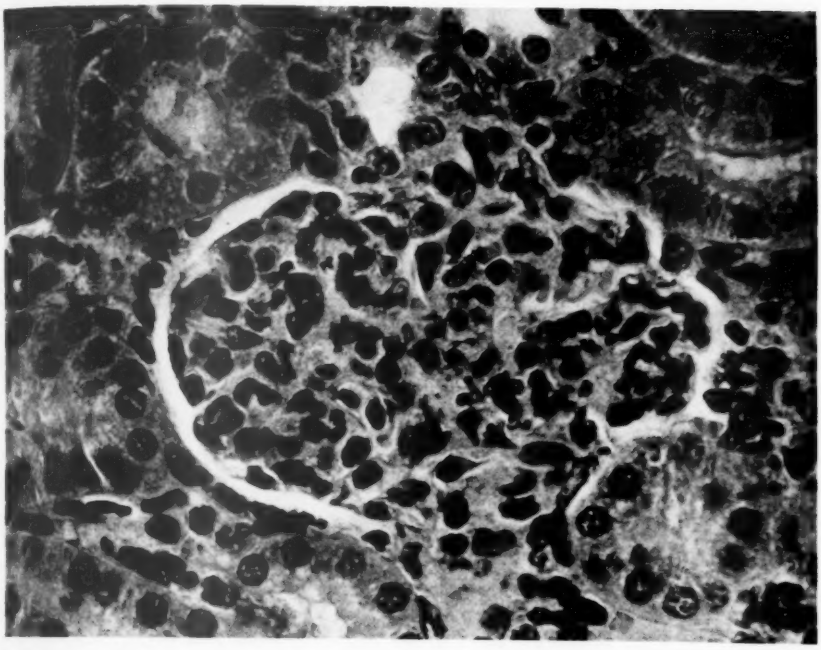
FIG. 5. Glomerulus from mouse given rabbit anti-BSA serum with excess BSA daily for 5 days. (Experiment IB). There is swelling and proliferation of glomerular cells and some mononuclear and slight neutrophil infiltration. × 450.

FIG. 6. Glomerulus from mouse given redissolved antigen-antibody complexes in 3 injections in 24 hours (ovalbumin antiovalbumin, Experiment IIIA). The glomerulus is hypercellular and bloodless. × 450.



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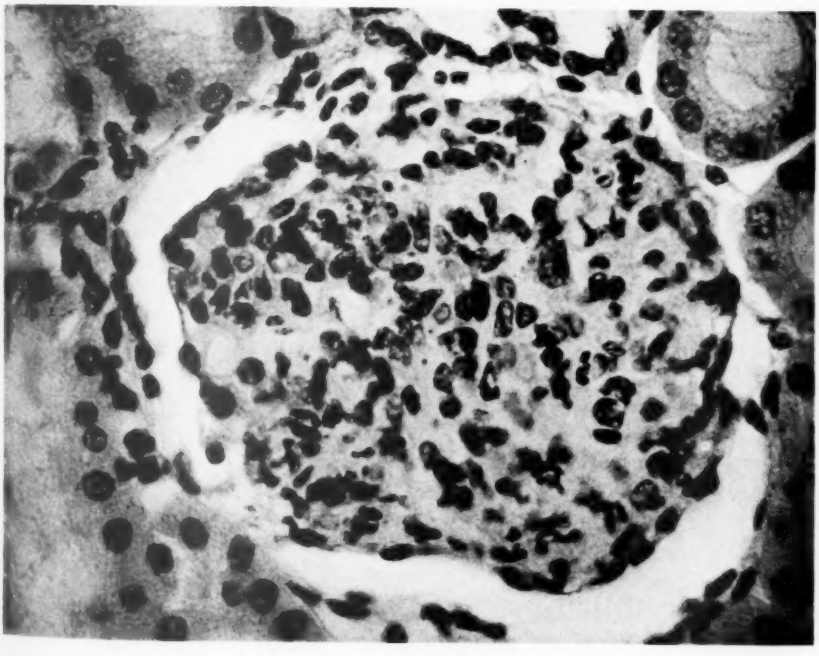

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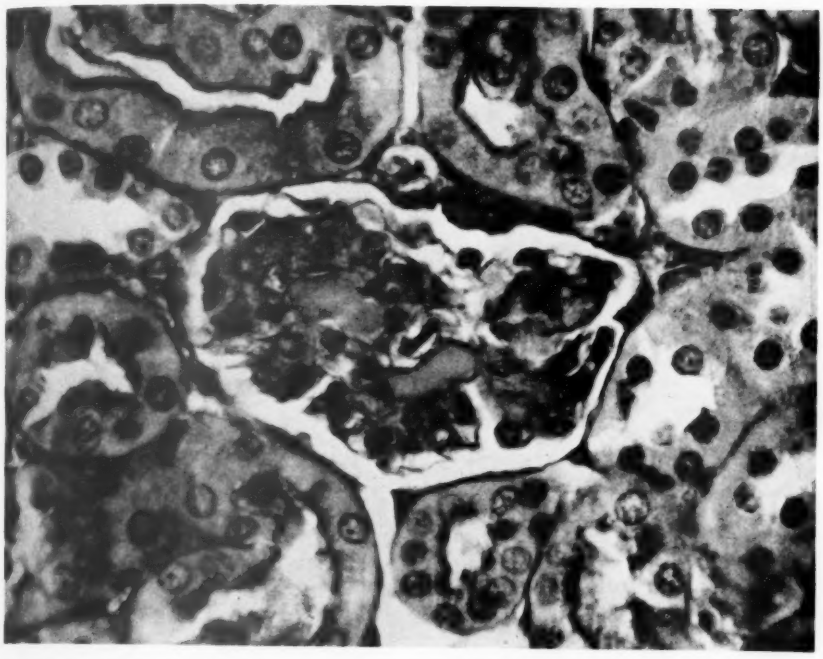
FIG. 7. Glomerulus from mouse given antiovalbumin rabbit serum with excess ovalbumin every 12 hours for 3 injections. There is homogeneous, eosinophilic, PAS-positive substance in glomerular capillaries. In this glomerulus, as in others containing a large amount of this material, there is no inflammation. Other glomeruli in this kidney showed acute glomerulitis. Periodic acid-Schiff (PAS) stain. × 450.

FIG. 8. Mitral valve from mouse given 3 injections of redissolved antigen-antibody complexes in 24 hours. (BSA-anti-BSA, Experiment IIIB). The valve leaflet is thickened and infiltrated with mononuclear cells, neutrophils and Anitschkow myocytes. × 125.



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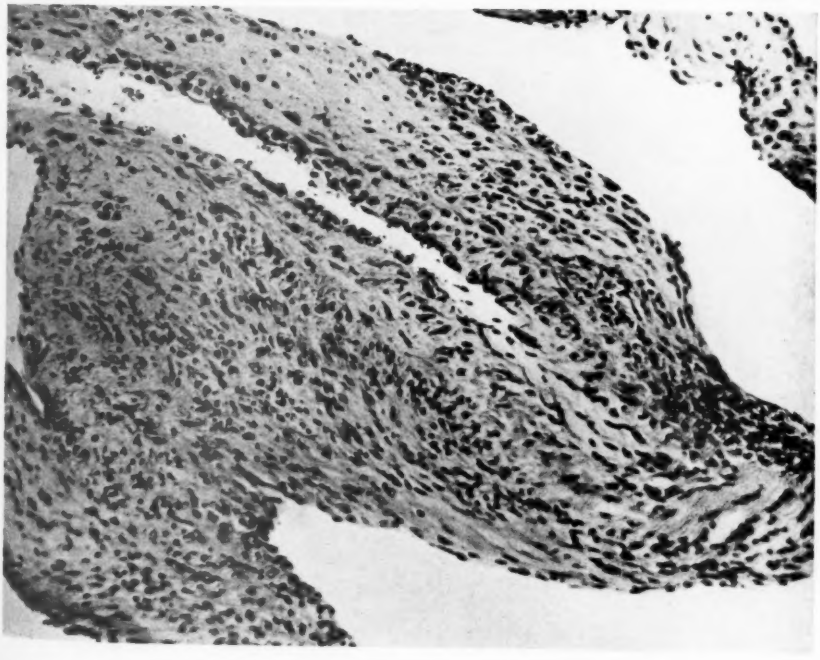

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FIG. 9. Coronary artery from mouse given 3 injections of redissolved antigen-antibody complexes in 24 hours. (BSA-anti-BSA, Experiment IIIB). There is segmental necrosis of the media and inflammation in all layers of the artery. × 175.

FIG. 10. Coronary artery, cut longitudinally, from mouse given anti-BSA serum with excess BSA daily for 5 days. (Experiment IB). There is necrosis of the media which has the staining characteristics of fibrinoid. × 175.

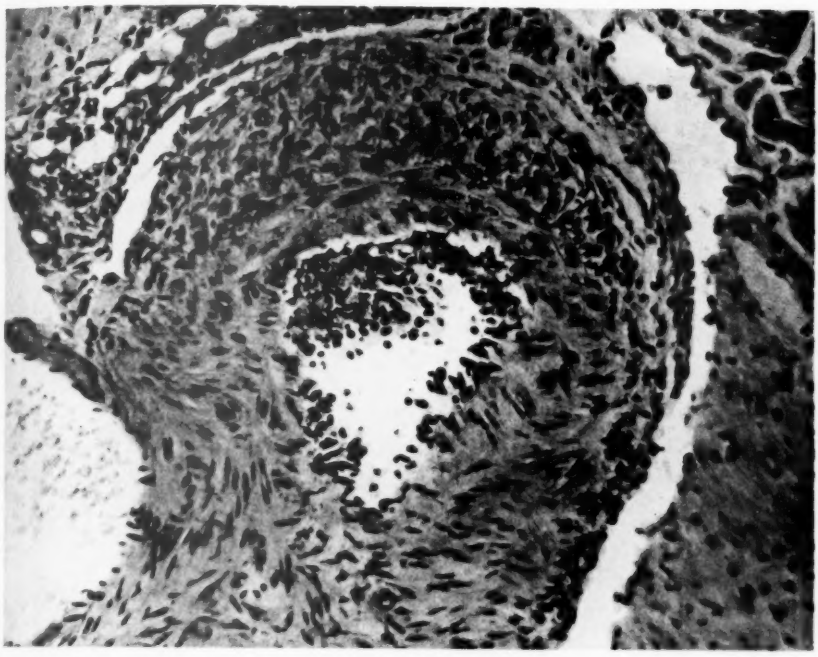

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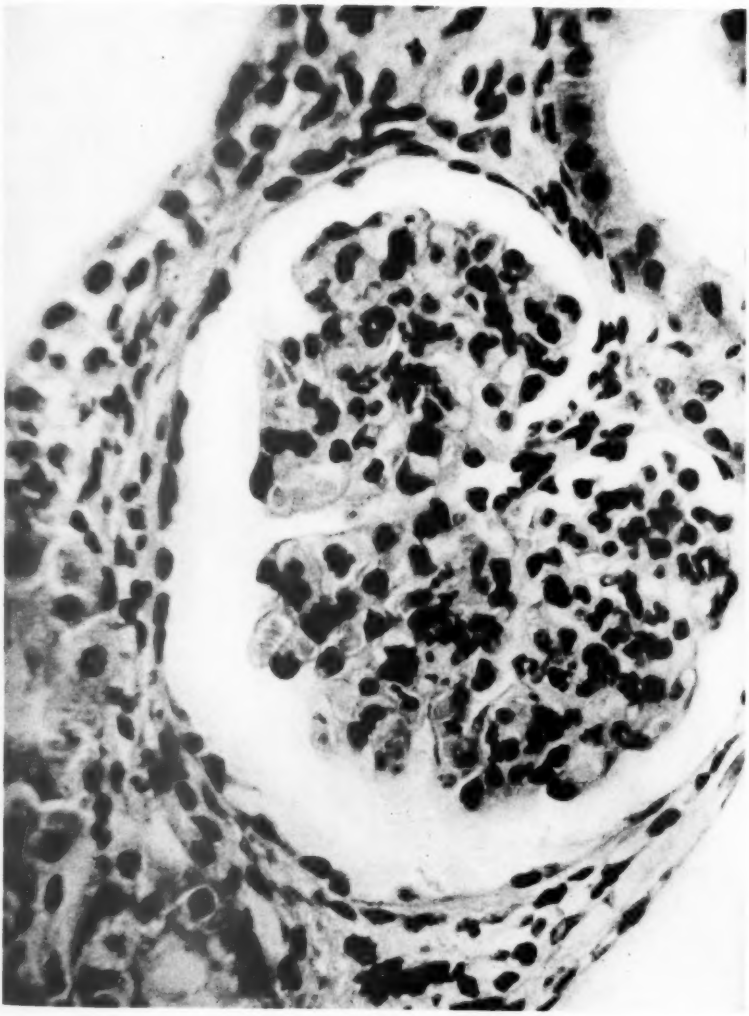
FIG. 11. Glomerulus from mouse given histamine and human gamma globulin daily for 5 days. There is hypercellularity and mild acute glomerulitis. Most of the glomeruli in this kidney were normal. × 450.



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PATHOGENESIS OF LESIONS INDUCED BY SEROTONIN:

NUTRITIONAL, VASCULAR, AUTORADIOGRAPHIC, AND COMPARATIVE STUDIES USING EPINEPHRINE *

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Patients with metastatic carcinoid tumor frequently demonstrate dyspnea, diarrhea, cyanosis, flushing, hepatomegaly, and right-sided heart failure.[1] Accompanied by acquired fibrotic lesions of the pulmonic and tricuspid valves[2] and peptic ulcer,[3] these combined phenomena have been designated the carcinoid syndrome.[3,4] Both the clinical and the pathologic features of the syndrome have been related to serotonin (5-hydroxytryptamine) since large quantities of this substance and its degradation product 5-hydroxyindolacetic acid appear in the blood and urine of patients with the syndrome, and because carcinoid tumors have been shown to contain[5] and to produce[6] serotonin.

Serotonin has been administered to experimental animals, chiefly rats, and up to the present, the lesions that were produced in acute experiments consisted of gastric mucosal erosions[7,8] and renal tubular and cortical necrosis.[9-12] In more prolonged experiments, similar renal lesions were induced, with scarring.[12,13] There were also necrosis of the tips of the tail and the digits,[13] ocular lenticular opacities,[13] and dermal fibrosis at injection sites.[13] The present series of experiments in rats are acute and chronic, in an attempt to study the manner in which serotonin and related substances produce lesions in the kidney, stomach, and skin. In addition, by the administration of serotonin to rats on a tryptophan deficient diet, an effort was made to produce valvular lesions of the heart.

MATERIAL

Approximately 350 male rats of the Sprague-Dawley strain (purchased from the Charles River Breeding Laboratories, Cambridge, Massachusetts), averaging 200 gm. in weight, with a range of 50 to 240 gm., comprised the experimental subjects. Two hundred rats were used in acute experiments, and 150 in chronic studies. Littermates were controls in all instances, in an approximate ratio of one control

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for each pair of test rats. Animals were housed in individual cages in an air-conditioned, constant temperature room. Pellets of Purina Laboratory Chow and water, *ad libitum*, were the standard diet, except in nutritional experiments.

Serotonin creatinine sulphate* and 5-hydroxytryptophan (California Foundation for Biochemical Research) were suspended in 2 to 5 ml. of physiologic saline, and injected subcutaneously or intraperitoneally, into demarcated areas, using 0.4, 1.68, 3.36, 6.72, and 30.24 mg. quantities. (Values are given in terms of the 5-hydroxytryptamine base, representing approximately 43 per cent of the serotonin creatinine sulphate). Control animals were given identical injections of physiologic saline or of other substances, as indicated in the experiments to be described.

Tissue, including that used for autoradiography, was ordinarily fixed in 10 per cent neutral buffered formalin at 4° to 6° C. In special instances, Zenker's solution with 5 per cent acetic acid, or freeze dry methods were used.

Sections were stained with hematoxylin and eosin, except those intended for autoradiography. The latter were either stained with Feulgen stain before applying film, or were stained with hematoxylin alone after the film had been developed. In addition, many special and histochemical stains were applied.

Experiments

Experiment 1. Heart

Attempts have been made previously to reproduce cardiac valvular lesions in rats by long-term administration of serotonin[13] and 5-hydroxytryptophan.[14] To date these have not been successful, despite the injection of serotonin subcutaneously twice daily for as long as one year. There is, thus, no experimental evidence to support the belief that the valvular fibrosis in patients with the carcinoid syndrome is caused by serotonin. It has long been suspected that certain manifestations of this syndrome may be the result of tryptophan or niacin deficiency.[15] The dietary source of serotonin and nicotinic acid is tryptophan. Since patients with extensive carcinoid dissemination utilize dietary tryptophan to produce serotonin, probably to the detriment of nicotinic acid formation, a deficiency of nicotinic acid may be induced. In studies of pellagra and "black tongue" in dogs,[16-18] valvular lesions have not been noted, although in two instances valvular lesions not definitely ascribable to the experimental diet were encoun-

* Supplied by Dr. George Berryman, Abbott Laboratories, and purchased from the California Foundation for Biochemical Research, Los Angeles, Calif.

tered.[18] To determine whether niacin or tryptophan deficiency alone, or in combination with an excess of serotonin in the blood, may give rise to valvular lesions, the following experiment was carried out.

Thirty-six male rats were fed *ad libitum* a tryptophan and niacin-deficient diet, beginning when they were 34 days old, and weighing 50 to 60 gm. The diet contained approximately 108 mg. of tryptophan per 100 gm. of diet and was composed of vitamin-free casein (Nutritional Biochemicals Corp., Cleveland, Ohio), 13 per cent; sucrose, 80 per cent; corn oil, 3 per cent; salts, 4 per cent (Salt Mixture W., Nutritional Biochemicals Corp.); L-cystine, 0.15 per cent. To this were added thiamine, 0.2 mg.; riboflavin, 0.3 mg.; pyridoxine, 0.25 mg.; dl-calcium pantothenate, 2.0 mg.; folic acid, 0.1 mg.; choline chloride, 100.0 mg.; *i*-inositol, 10.0 mg.; and biotin, 0.01 mg., per 100 gm. of diet. Cod liver oil was also added to give each rat approximately 2 drops per week.

Twelve of the rats received subcutaneous injections of 3.36 mg. of 5-hydroxytryptamine twice daily for 90 consecutive days; 12 received injections of saline, and 12 received the diet alone. As further controls, 24 littermates received a regular diet of Purina Laboratory Chow and water; 12 of these received injections of serotonin as in the test group above, 6 received saline injections, and 6 received no injections. Twelve of the control rats were pair-fed with 12 of the test rats to assess the role of caloric intake. Animals fed the deficient diet failed to gain weight. They exhibited an average weight of 168 gm. at the end of the 90-day period as compared with a weight of 434 gm. in the control rats fed the stock pellet diet. Because of this, it was considered desirable to supplement the diets with tryptophan; 1 gm. of DL-tryptophan was added to the diet of each rat 5 times during the 90-day period.

The animals fed the deficient diet without serotonin injection showed atrophy of the skin, thymus, testis, and occasionally, minimal fatty vacuolization of the liver. Also encountered were focal myocardial and renal necrosis with calcareous deposits. The cardiac lesions have been reported previously in rats on tryptophan-deficient diet.[19,20] The group of animals in which serotonin supplemented the deficient diet also showed renal cortical and tubular necrosis, and dermal thickening at injection sites. Well defined fibrotic lesions of the heart valves were not found, however. Animals fed the deficient diet occasionally showed slight thickening and apparent cellular disarrangement of both the right and left atrial endocardium, but this lesion was not constant (10 of 36 animals). It was so slight as to be of questionable significance, although similar alterations were not found in control animals.

Experiment 2. Kidney

In acute studies, renal tubular necrosis was observed within 6 hours after a single injection of 3.36 mg. or more of 5-hydroxytryptamine base, whether given subcutaneously or intraperitoneally. To study the pathogenesis of these lesions, undiluted India ink was injected into the renal arteries and veins of 60 rats 1 to 2 hours after a single injection of 3.36 mg. or more of serotonin. Animals were anesthetized with ether, the abdomen was entered anteriorly, and, with a mercury manometer, 3-way stopcock, a 10-ml. syringe, and a 20-gauge needle, in a 3 minute period 3 ml. of India ink was injected into the left renal artery and 3 ml. into the left renal vein at an approximate pressure of 110 to 130 mm. of Hg. The animals were then sacrificed immediately by decapitation, and the kidneys incised twice parallel to the long axis to insure adequate fixation. The organs were immersed in 10 per cent neutral buffered formalin at 4° C. for 24 hours. Frozen sections were cut at 75 to 100 μ thickness, and mounted unstained in polyvinyl pyrrolidone.[21] Additional animals were sacrificed at later intervals, 4 to 24 hours after identical injections of serotonin, and histologic sections of the kidneys stained with hematoxylin and eosin were examined. As shown in Figures 1 to 4, ischemia of portions of the renal tubules was demonstrated by the India ink injections. This phenomenon preceded the appearance of tubular necrosis (Figs. 5 to 7). The ischemia was usually most marked in the regions supplied by post-glomerular arterioles (Fig. 4). Epinephrine, 0.2 ml. of 1:1,000 concentration, 1 mg. per kg., was injected subcutaneously in additional animals, and in these, diffuse zones of ischemia were demonstrated by India ink injection. Here, too, tubular and, in some cases, cortical necrosis appeared later (Figs. 7 and 8).

Experiment 3. Skin

In previous studies, it was noted that serotonin creatinine sulphate, when injected subcutaneously in 3.36 mg. quantities of the base twice daily, in rats for periods up to one year, resulted in dermal fibrosis at the injection sites.[22] To further test this observation, similar quantities of serotonin were injected twice daily for 73 days into 6 rats. Additional rats, 6 in each group, were given injections of creatinine, 8 mg.; 5-hydroxyindolacetic acid, 8 mg.; tryptophan, 8 mg.; methionine, 8 mg; and 0.2 ml. 1:1,000 epinephrine (1 mg. per kg.) in a similar manner.

At the end of 73 days, only 3 groups showed skin lesions at the injection sites. These were present in all of the animals receiving serotonin creatinine sulphate, 5-hydroxytryptophan, and epinephrine (Figs. 9 to 12). The alterations were similar in the 3 groups, if not identical, ex-

cept that those due to serotonin were the most advanced. In the control groups, the skin exhibited no thickening (Fig. 10).

Experiment 4. Stomach

Intraperitoneal administration of very large doses of 5-hydroxytryptophan[7] and serotonin[8] have been shown to produce gastric mucosal erosions in the rat in 3 to 4 hours. With smaller doses, injected subcutaneously over a one year period, however, gastric erosions were not found.[13] In the following experiments, the administration of epinephrine and of 5-hydroxytryptophan by the intraperitoneal route was followed by similar gastric mucosal erosions. The dosages used were 300 to 350 mg. of 5-hydroxytryptophan per kg. and 0.2 ml. of 1:1,000 solution of epinephrine (1 mg. per kg.). Animals were sacrificed 3 hours later. Among the rats receiving epinephrine, 14 of 17 (82 per cent) developed gastric erosions. Twenty-seven of 41 animals (66 per cent) receiving 5-hydroxytryptophan exhibited similar lesions. Histologically, the erosions were identical in both instances, except that those due to epinephrine were often larger (Figs. 13 to 15).

Experiment 5. Autoradiography

In autoradiographic studies, approximately 30 rats received single subcutaneous injections of 3.36 mg. of 5-hydroxytryptamine. In addition 6 rats were given injections of 0.2 ml. of 1:1,000 epinephrine, and 30 rats received physiologic saline. Twenty-four hours after the injections, a single injection of tritiated thymidine (Schwarz Laboratories, Inc., Mount Vernon, New York) was administered intraperitoneally. The dosage was 1 μc. per gm. of body weight; all preparations had a specific gravity of 360 mc. per mM. The rats were sacrificed 4 hours later by decapitation. Similar injections of tritiated thymidine were given to 6 rats that had received 5-hydroxytryptamine, 3.36 mg., subcutaneously twice daily for 14 days, and to 6 rats that had been given saline injections for a like period. Tissues of these animals were sectioned and examined by conventional histologic stains, and by preparation of autoradiographs, using a stripping film technique (Film AR. 10, Kodak Ltd., Kingsway, London, England).[23] Histologic sections of 6 μ thickness were generally exposed to stripping film for 4 weeks; development was carried out in a Kodak D-19 developer for 7 minutes, and Kodak acid fixer for 10 minutes. Sections were usually mounted with polyvinyl pyrrolidone (PVP; Antara Chemicals, New York City), which permitted permanent mounting directly from water, without dehydration.

The purpose of the autoradiographic studies was to determine

whether serotonin caused a proliferative tissue response, irrespective of that which might follow its ischemic and necrotizing effects. Tritiated thymidine was especially suitable because the short beta emission of the tritium label permitted the detection of individual nuclei bearing the isotope,[24] and the thymidine was incorporated only into deoxyribonucleic acid (DNA).[25,26] The DNA of cells is confined chiefly to the nucleus,[27,28] and is renewed only during preparation of cells for division,[29,30] so that by this method, only cells synthesizing DNA at the time that the tritiated thymidine is injected will take up the substance. All tissues of the body were examined, with special attention to the heart and its valves, lung, liver, skin, brain, gastrointestinal tract, kidney, adrenal, spleen, thymus, and tongue. Only in the necrotizing renal lesions was there unequivocal evidence of a proliferative response as shown by an increase in labeled nuclei. This was the case, also, in the rats receiving epinephrine. A slight indication of proliferation in hepatic cells, unaccompanied by necrosis, is still under investigation.

Discussion

The present series of experiments are interpreted to show that serotonin and related substances induce lesions in rats by producing ischemia and necrosis. Autoradiographic studies with tritiated thymidine indicate that serotonin probably has no direct stimulatory effect upon cellular proliferation.

The nature of the renal lesions which follow serotonin administration leave little doubt that ischemia is an important factor, if not the only one, in their development. Allowing for variations, in view of the fact that approximately 10 per cent of any group of rats given single doses of serotonin did not develop significant renal tubular necrosis or evidences of zonal renal ischemia as determined by India ink injections, the lesions illustrated were found consistently. The observation that similar, if not identical, lesions were observed in the kidneys, stomach, and skin, following the administration of epinephrine, further suggests that the tissue alterations ascribed to large doses of serotonin are the result of ischemia. It is difficult to test this explanation further because of the large and nonphysiologic doses which were necessary to produce well defined lesions. For example, we have tried to prevent the ischemic actions of serotonin and epinephrine by first treating the animals with such vasodilators as sodium nitrite and ergotrate, the latter being known to oppose the vasoconstrictive action of epinephrine.[31] These studies have not had consistent results, since large dosages of the vasodilators are required to oppose the large amounts of serotonin and epinephrine utilized. The rats developed near fatal or even fatal hypo-

tension from the vasodilators alone. Erspamer, however, in physiologic experiments investigating renal blood flow and urine formation, successfully blocked the vasoconstrictive effects of serotonin by pretreatment with vasodilators.[32]

The induction of gastric mucosal erosions in rats given serotonin has permitted a certain amount of speculation that serotonin may be an important factor in the pathogenesis of peptic ulcer in human subjects. Because of the large, nonphysiologic doses required to produce the experimental lesion, however, such a relationship appears to be unlikely, although it is true that the incidence of peptic ulcer is quite high in patients with the carcinoid syndrome.[3,7]

Further attention to experimental production of cardiac valvular lesions analogous to those in the carcinoid syndrome appears justified, not only for its elucidation in this disease, but for the insight that may be gained into valvular lesions in other conditions such as rheumatic valvulitis, and nonspecific degenerative alterations so frequently found in persons dying with disorders not primarily related to the heart.[3,33]

Summary and Conclusions

A series of experiments is described in which the morphologic effects of serotonin and related substances have been investigated in rats.

Administration of serotonin to rats fed a diet deficient in niacin and tryptophan for 90 days did not result in cardiac valvular fibrosis.

Investigation of the renal vascular system by the injection of India ink revealed an identity of lesions induced by serotonin and epinephrine. This was also the case in respect to lesions produced in the skin and stomach by these two agents and was considered to be a reflection of focal ischemia.

As determined by autoradiographic studies with the tritiated DNA precursor, thymidine, serotonin possessed no direct stimulating effect upon cellular proliferation. Proliferative activity which did occur in renal tubules was attributable to a response to ischemic necrosis and not to serotonin.

REFERENCES

1. Thorson, A.; Biörck, G.; Björkman, G., and Waldenström, J. Malignant carcinoid of the small intestine with metastases to the liver, valvular disease of the right side of the heart (pulmonary stenosis and tricuspid regurgitation without septal defects), peripheral vasomotor symptoms, bronchoconstriction, and an unusual type of cyanosis: a clinical and pathologic syndrome. *Am. Heart J.*, 1954, **47**, 795–817.

2. Isler, P., and Hedinger, C. Metastasierendes Dünndarmcarcinoid mit schweren vorwiegend das rechte Herz betreffenden Klappenfehlern und Pulmonalstenose—ein eigenartiger Symptomenkomplex? *Schweiz. med. Wchnschr.*, 1953, **83**, 4–7.

3. MacDonald, R. A. A study of 356 carcinoids of the gastrointestinal tract: report of four new cases of the carcinoid syndrome. *Am. J. Med.*, 1956, **21**, 867–878.

4. Sjoerdsma, A.; Weissbach, H., and Udenfriend, S. Simple test for diagnosis of metastatic carcinoid (argentaffinoma). (Letter to the editor.) *J.A.M.A.*, 1955, **159**, 397.

5. Lembeck, F. 5-Hydroxytryptamine in a carcinoid tumour. (Letter to the editor.) *Nature, London*, 1953, **172**, 910–911.

6. Sjoerdsma, A.; Weissbach, H.; Terry, L. L., and Udenfriend, S. Further observations on patients with malignant carcinoid. *Am. J. Med.*, 1957, **23**, 5–15.

7. Haverback, B. J., and Bogdanski, D. F. Gastric mucosal erosion in the rat following administration of the serotonin precursor, 5-hydroxytryptophan. *Proc. Soc. Exper. Biol. & Med.*, 1957, **95**, 392–393.

8. Hedinger, C., and Veraguth, F. Magengeschwüre bei Ratten unter Behandlung mit 5-Hydroxytryptamin. *Schweiz. med. Wchnschr.*, 1957, **87**, 1175–1176.

9. Page, E. W., and Glendening, M. B. Production of renal cortical necrosis with serotonin (5-hydroxytryptamine). (Abstract) *Am. J. Med.*, 1955, **19**, 285.

10. Hedinger, C., and Langemann, H. Neirenschädingungen mit Rindennekrosen bei Ratten unter Behandlung mit 5-Oxytryptamin: Weiterer experimenteller Beitrag zur Frage der endokrinen Aktivität der Karzinoide. *Schweiz. med. Wchnschr.*, 1955, **85**, 541–544.

11. MacDonald, R. A.; Robbins, S. L., and Mallory, G. K. Morphologic effects of serotonin: relation to cardiac lesions of the carcinoid syndrome. (Abstract) *Clin. Res.*, 1958, **6**, 23.

12. Fiore-Donati, L., and Erspamer, V. Studies on the nephrotoxicity of 5-hydroxytryptamine (enteramine) in the rat. *Am. J. Path.*, 1957, **33**, 895–917.

13. MacDonald, R. A.; Robbins, S. L., and Mallory, G. K. Morphologic effects of serotonin (5-hydroxytryptamine). *A.M.A. Arch. Path.*, 1958, **65**, 369–377.

14. Davidson, J.; Sjoerdsma, A.; Loomis, L. N., and Udenfriend, S. Studies with the serotonin precursor, 5-hydroxytryptophan, in experimental animals and man. *J. Clin. Invest.*, 1957, **36**, 1594–1599.

15. McNeely, R. G. D., and Jones, N. W. Secondary pellagra caused by multiple argentaffin carcinoma of the ileum and jejunum. *Gastroenterology*, 1946, **6**, 443–448.

16. Denton, J. The pathology of pellagra. *Am. J. Trop. Med.*, 1925, **5**, 173–210.

17. Denton, J. A study of the tissue changes in experimental black tongue of dogs compared with similar changes in pellagra. *Am. J. Path.*, 1928, **4**, 341–352.

18. Lillie, R. D. Pathology of experimental blacktongue. *Bull. Nat. Inst. Health*, 1933, **162**, 13–21.

19. Scott, E. B. Histopathology of amino acid deficiencies. IV. Tryptophan. *Am. J. Path.*, 1955, **31**, 1111–1129.

20. Adamstone, F. B., and Spector, H. Tryptophan deficiency in the rat. Histologic changes induced by forced feeding of an acid-hydrolyzed casein diet. *Arch. Path.*, 1950, **49**, 173–184.

21. Burstone, M. S. Polyvinyl pyrrolidone as a mounting medium for stains for fat and for azo-dye procedures. *Am. J. Clin. Path.*, 1957, **28**, 429–430.

22. MacDonald, R. A.; Robbins, S. L., and Mallory, G. K. Dermal fibrosis following subcutaneous injections of serotonin creatinine sulphate. *Proc. Soc. Exper. Biol. & Med.*, 1958, **97**, 334–337.

23. Doniach, I., and Pelc, S. R. Autoradiographic technique. *Brit. J. Radiol.*, 1950, **23**, 184–192.

24. Fitzgerald, P. J.; Eidinoff, M. L.; Knoll, J. E., and Simmel, E. B. Tritium in radioautography. *Science*, 1951, **114**, 494–498.

25. Friedkin, M.; Tilson, D., and Roberts, D. Studies of deoxyribonucleic acid biosynthesis in embryonic tissues with thymidine-C^{14}. *J. Biol. Chem.*, 1956, **220**, 627–637.

26. Reichard, P., and Estborn, B. Utilization of desoxyribosides in the synthesis of polynucleotides. *J. Biol. Chem.*, 1951, **188**, 839–846.

27. Vendrely, R. The Deoxyribonucleic Acid Content of the Nucleus. In: The Nucleic Acids: Chemistry and Biology. Chargaff, E., and Davidson, J. N. (eds.). Academic Press, Inc., New York, 1955, Vol 2, pp. 155–180.

28. Thorell, B. Nucleic Acids in Chromosomes and Mitotic Division. In: The Nucleic Acids: Chemistry and Biology. Chargaff, E., and Davidson, J. N. (eds.). Academic Press, Inc., New York, 1955, Vol. 2, p. 185.

29. Goldthwait, D. A., and Bendich, A. Effects of a folic acid antagonist on nucleic acid metabolism. *J. Biol. Chem.*, 1952, **196**, 841–852.

30. Hevesy, G. Radioactive Indicators. Their Application in Biochemistry, Animal Physiology, and Pathology. Interscience Publishers, Inc., New York, 1948, pp. 325–330.

31. Beckman, H. Pharmacology in Clinical Practice. W. B. Saunders Co., Philadelphia, 1952, p. 125.

32. Erspamer, V. Pharmacological studies on enteramine (5-hydroxytryptamine). IX. Influence of sympathomimetic and sympatholytic drugs on the physiological and pharmacological actions of enteramine. *Arch. Internat. Pharmacodyn.*, 1953, **93**, 293–316.

33. MacDonald, R. A., and Robbins, S. L. The significance of nonbacterial thrombotic endocarditis: an autopsy and clinical study of 78 cases. *Ann. Int. Med.*, 1957, **46**, 255–273.

[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Kidney, 200 gm. control rat. India ink injected via the renal artery. There is normal filling of glomeruli and peritubular vessels. Unstained, formalin-fixed, frozen section. × 30.

FIG. 2. Kidney, 200 gm. rat. India ink injected at a pressure of approximately 200 mm. of Hg via the renal artery one hour after a single subcutaneous injection of 5-hydroxytryptamine (24 mg. per kg.). Note zones, including the outer portion of the cortex, where there is little or no filling of vessels. Unstained, formalin-fixed, frozen section. × 30.

FIG. 3. Kidney, 200 gm. rat. India ink injected at a pressure of approximately 110 mm. of Hg via the renal artery, one hour after a single subcutaneous injection of 5-hydroxytryptamine (24 mg. per kg.). Note the zones of marked ischemia; intralobular arteries can be traced to afferent arterioles and glomeruli, but post-glomerular arterioles and capillaries do not fill. Unstained, formalin-fixed, frozen section. × 100.

FIG. 4. Kidney, 200 gm. rat. A single subcutaneous injection of 5-hydroxytryptamine (34 mg. per kg.) has been administered and the rat sacrificed 24 hours later. Note extensive tubular necrosis, affecting chiefly the proximal tubules, with homogeneous cytoplasmic appearance, and disappearance of nuclei. The outer rim of the cortex is also affected. Hematoxylin and eosin stain. × 200.

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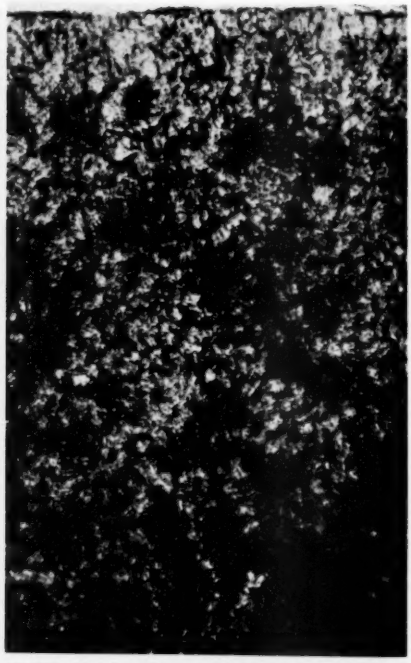

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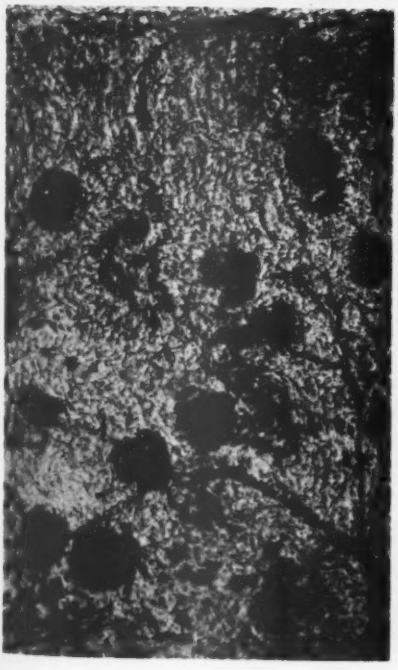

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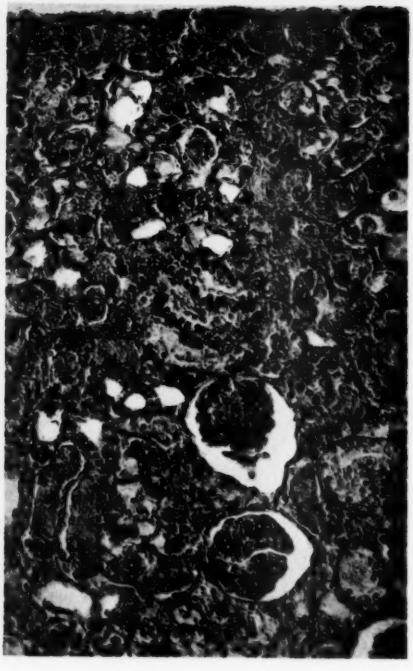

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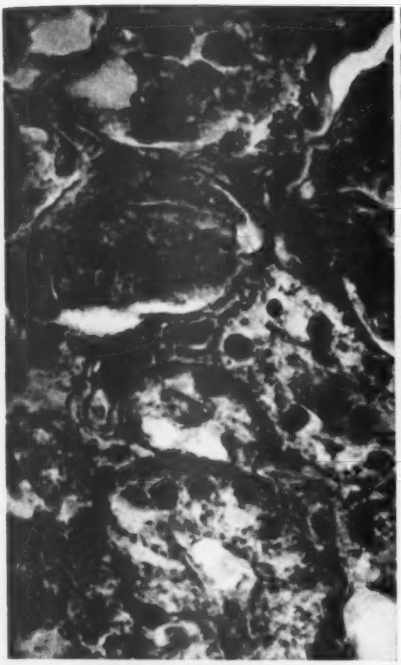
FIG. 5. Kidney, 200 gm. rat. A single injection of 5-hydroxytryptamine (174 mg. per kg.) has been given and the rat sacrificed 5 hours later. Note renal tubular necrosis, most marked along the left edge of the photograph. Hematoxylin and eosin stain. × 800.

FIG. 6. Kidney, 200 gm. rat. A single intraperitoneal injection of 5-hydroxytryptophan (350 mg. per kg.) has been given and the rat sacrificed 5 hours later. Note necrosis of the tubules adjacent to a glomerulus. Hematoxylin and eosin stain. × 800.

FIG. 7. Kidney, 200 gm. rat. A single intraperitoneal injection of 0.2 ml. of 1:1,000 epinephrine (1 mg. per kg.) has been given and the rat sacrificed 5 hours later. Note the early necrosis of tubules adjacent to a glomerulus. Hematoxylin and eosin stain. × 800.

FIG. 8. Kidney, 200 gm. rat. Subcutaneous injections of 1:1,000 epinephrine (1 mg. per kg.) have been given twice daily for 2 days. Note extensive infarction of the cortex in the right half of the photograph. Hematoxylin and eosin stain. × 100.



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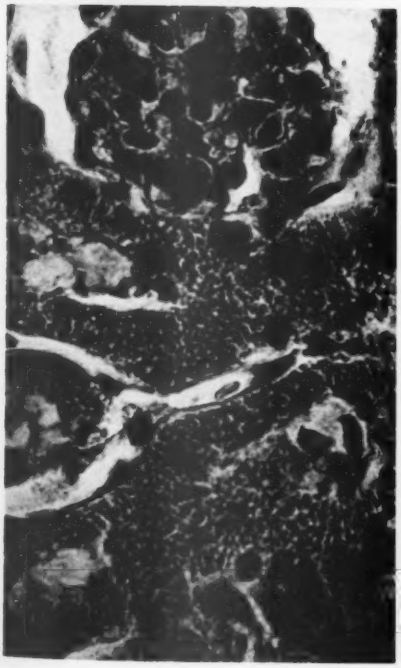

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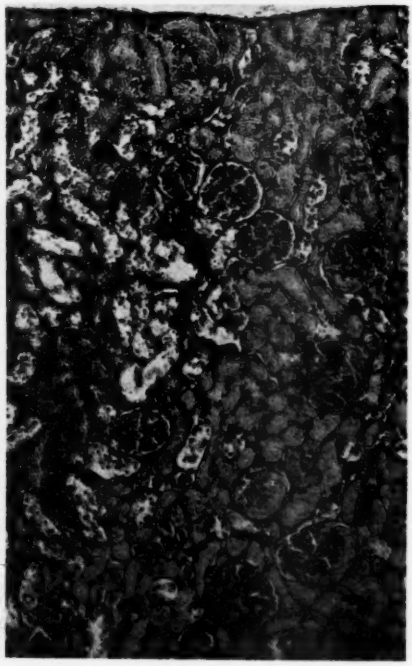

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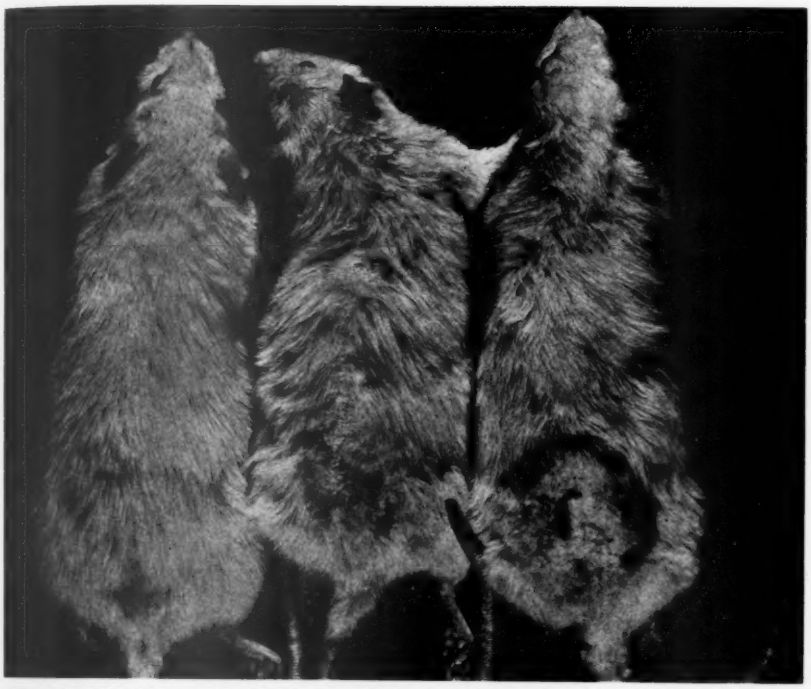
FIG. 9. Three rats received subcutaneous injections twice daily for 38 consecutive days. Injections were administered into an identical region of the back, above the tail. The rat on the left received creatinine, the one in the center, serotonin, and the animal on the right, epinephrine. Note the normal skin in the animal given creatinine and the loss of hair and dermal thickening at injection sites of the animals receiving serotonin and epinephrine.

FIG. 10. Skin of rat in Figure 9 which received creatinine injections. Note normal, loose dermis and thin epidermis. Hematoxylin and eosin stain. × 200.

FIG. 11. Skin of rat in Figure 9 which received serotonin injections. Note thick dermis with dense collagenous fibers, and thickened epidermis. Hematoxylin and eosin stain. × 300.



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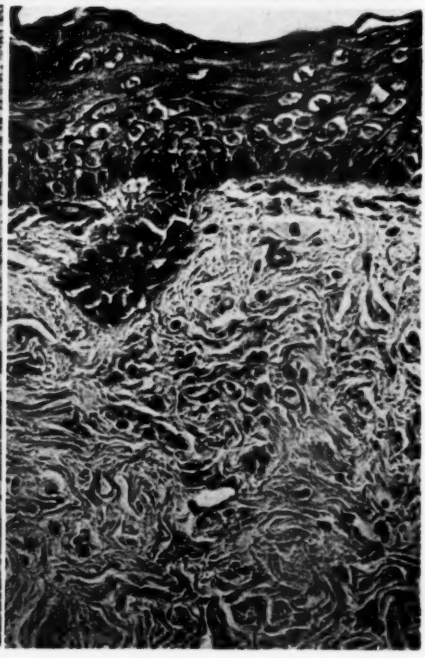

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FIG. 12. Skin of rat in Figure 9 which received epinephrine injections. Note thickened dermis and epidermis, similar to that shown in Figure 11. Hematoxylin and eosin stain. × 200.

FIG. 13. Stomach and duodenum of a rat given 400 mg. per kg. of 5-hydroxytryptophan intraperitoneally and sacrificed 3 hours later. Note mucosal erosions in glandular portion of stomach.

FIG. 14. Stomach and duodenum of a rat given 0.2 ml. of 1:1,000 epinephrine (1 mg. per kg.) intraperitoneally, and sacrificed 3 hours later. Note large mucosal erosion.

FIG. 15. Gastric mucosa of a rat given 1 mg. per kg. of epinephrine intraperitoneally and sacrificed 3 hours later. Note sharp demarcation between partially necrotic mucosa on left, and intact mucosa on right. Hematoxylin and eosin stain. × 200.

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EXPERIMENTAL PRODUCTION OF CONGENITAL MALFORMATIONS IN RATS BY SALICYLATE POISONING*

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Many methods are now available which permit experimental production of congenital malformations of the central nervous system in mammals,[1] and it may seem unnecessary to add yet another procedure to this long list. Actually, these techniques and their results are not so uniform as they may appear to those not working in the field of experimental teratology. Methods differ in the yield of abnormal young, in the types of malformation produced, and in the mechanisms involved. There are individual and species differences, and there are variations due to timing and specificity of the injury.

We have attempted the experimental production of congenital malformations by large doses of salicylates because the pathologic physiology of salicylate poisoning has been thoroughly investigated and because it seemed possible that some of the known metabolic effects of salicylates could be specifically responsible for disturbed embryogenesis. Before such investigations could be done, it was necessary to ascertain in preliminary experiments whether salicylates had any teratogenic effects at all. In the course of a pilot study, a number of unusual terata was obtained. These may be of sufficient interest to merit a special report.

METHODS

One hundred and sixteen female rats, weighing about 170 to 200 gm., were obtained from commercial breeders. The rats were mated and the day on which sperm was found in vaginal smears was considered the first day of pregnancy. On the ninth, tenth, or eleventh day of pregnancy, these females received single subcutaneous injections of methyl salicylate (synthetic, Merck & Co., Inc., Rahway, New Jersey) in doses of from 0.1 to 0.5 cc. Most of the females lost about 15 to 25 gm. after this treatment, but some recovered and regained the weight lost. Twenty-six females died, and 47 resorbed their young after treatment. The 43 rats that remained pregnant regained the weight lost within 3 or 4 days and continued gaining until they were sacrificed on the 21st day. A total of 298 young were obtained in this way.

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Another 43 females received single, subcutaneous injections of sodium salicylate (Eli Lilly and Co., Indianapolis, Indiana) in doses of 60 to 180 mg. on the ninth, tenth, or eleventh day of pregnancy. Six rats which had been given more than 120 mg. died within 24 hours after the injection. Of the remaining 37 rats, 24 resorbed their young, and only 13 carried their litters to the 21st day of pregnancy, when they were sacrificed. One hundred young were removed from these females.

Results

Of the 298 young obtained from mothers which had received injections of methyl salicylate, 45 were externally abnormal. Of the 253 young which appeared externally normal, 201 were cleared by the Schultze-Dawson method,[2] and 75 showed skeletal anomalies. Fifty-two young that appeared externally normal were fixed in Bouin solution for later sectioning. Thus, at least 120 (40.3 per cent) of the 298 young were abnormal.

Of the 100 young from mothers treated with sodium salicylate, 15 were externally abnormal. Of 85 young which were externally normal, 66 were cleared and 11 had skeletal anomalies. Thus, of the 100 young, at least 26 were abnormal.

One hundred and five females were used as controls, and 59 had litters producing a total of 484 young. None of the young had external malformations. Fifty-nine young were cleared and were found to be normal with the exception of 3 which had "wavy ribs."

The most interesting congenital malformations were found in the group treated with methyl salicylate. There were 12 young that had craniorachischisis, a striking malformation not seen frequently in previous teratologic experiments (Figs. 2, 3 and 10). Five of these monsters also had gastroschisis with eventration of the stomach, liver and intestine. The protruding liver, which was definitely oversized and subdivided into many lobes, extended to beneath the forepaws, while the protruding intestine and stomach were situated low on the right and on the left side respectively, above the hind legs. The lower half of the body was markedly shortened. Because of this shortness, the head of the young with craniorachischisis appeared relatively large (Fig. 10). It was connected to the trunk by a thick and short neck. The normal neck groove between the mandible and the chest was absent, and the skin passed from the lower jaw directly to the chest. A protruding tongue was seen in most of these animals. While in normal young the forehead rises behind the snout, in the abnormal young the head was completely flat. Behind the eyes the brain pro-

truded, the exposed parts sitting in the manner of a skullcap on the back of the head. There it was directly connected with the exposed medullary plate (Figs. 2, 3 and 10). The back, which is convex in the normal newborn rat, was either straight or concave. The open neural plate, clearly divided by a median sulcus corresponding to the embryonic neural groove, narrowed in its downward course and ended above the base of the tail. The exposed brain was well preserved, and one could recognize the hemispheres of the forebrain, the midbrain and the hindbrain, but the cerebellum was absent. In the back the skin defect was larger than the exposed neural plate. On the smooth and glistening parallel strips interposed between the margins of the skin and the nervous tissue, segmentally arranged elevations could be discerned representing the lateral portions of the unfused vertebrae (Fig. 2). The open neural plate was straight in some and twisted in other specimens (Fig. 3).

The brain was studied in frontal histologic sections and will be described in rostral-caudal sequence. The entire brain was displaced backward. While in normal animals the rostral ends of the olfactory lobes extend forward beyond the ocular region and the pallium beyond the exit of the optic nerve (Fig. 4), in the abnormal young the olfactory lobes appeared at the level of the exit of the optic nerve and the pallium behind the posterior pole of the *bulbus oculi* (Fig. 5). The olfactory lobes were deformed and appeared triangular in cross sections. In sections showing the rostral end of the pallium appearing over the rhinencephalon (Fig. 5), the brain broke through the vault of the cranium but remained covered by skin. In sections through the junction of the ventriculus of the olfactory lobe with the anterior horn of the lateral ventricle, all the layers of the skin narrowed, and the brain reached the surface of the head. From here on, the exposed portions of the brain were covered only by a thin layer of *stratum corneum*. The gross architecture of the brain was maintained. The lateral ventricles were limited medially by the *septum pellucidum* and laterally by the caudate nucleus. The *nucleus lentiformis,* the internal capsule, the *corpus callosum,* and the cortex could be identified, but the finer architecture of these structures was severely disturbed. The position of the olfactory tract was peculiarly anomalous, and, instead of lying on the ventrolateral surface, it was enclosed by a fold formed by the rhinencephalon and the pallium. A section through the optic chiasm showed that the two hemispheres were connected by the *corpus callosum* and by the anterior commissure. There was no trace of a third ventricle in sections rostral to the foramens of Monro, which

connected the lateral ventricles with each other but not with the third ventricle. The solid connection of the two halves of the diencephalon contained irregular and asymmetrical nuclear masses.

The pituitary gland was well developed, and its structure appeared normal. Although most of the normal cell masses could be identified, they appeared distorted and increased in density. The dorsal portion of the third ventricle and its *tela chorioidea* were absent, and in their place a triangular process, consisting of solid nervous tissue, protruded between small and deformed Ammon's horns into the median sagittal fissure (Fig. 8). In the hypothalamic region a rudimentary third ventricle could be recognized, and a second smaller central opening was seen dorsally between the thalamic masses. In the next sections the triangular dorsal process became larger and penetrated between the hemispheres to reach the vertex of the head. The pineal body and the pineal recess were absent. The lateral ventricles were enlarged and partly filled with blood in their posterior aspects. The portion of the midbrain situated on the vertex of the head was covered by blood and *stratum corneum* which showed signs of beginning erosion. The brain lay without cutaneous or osseous covers over its convexity, on the flat floor of the cranium. The rudiments of the third ventricle ended blindly, and there was no aqueduct. In the subsequent sections the distal end of the hemispheres flanked portions of the midbrain and hindbrain, but the cerebellum was absent and the fourth ventricle not recognizable. The medulla oblongata lay open on the back of the neck, divided by a raphe into a right and a left half.

The trunk and the spinal central nervous system were studied in sections at right angle to the spinal axis. One could follow the gradual transformation of the dorsally open medulla into an open neural plate. This plate, which lay over the trough formed by defective vertebral bodies and open neural arches, was ventrally subdivided by the anterior median sulcus (Fig. 9). Lateral to the sulcus the ventral funiculi were clearly outlined, and the anterior horns could be recognized. The equivalents of the lateral and posterior horns formed large cell masses occupying the lateral portions of the plate. There was no central canal. The posterior surface was well defined and represented by a dense, intensely stained membrane, which was connected laterally to a thin layer of *stratum corneum* derived from the skin. Normal skin, which covered all the ventral parts of the body, was absent over the entire neural plate and also over the open neural arches. In the midline, dorsal to the anterior median sulcus, ependymal

cells could be clearly recognized at the dorsal surface of the neural plate. In spite of its abnormal development, the size of the neural plate was, in cross section, about twice that of a normal spinal cord.

The subarachnoid space ventral to the neural plate was much enlarged so that the nervous tissue was pushed dorsad beyond the level of the skin. In sections through the upper thoracic region the neural plate assumed the shape of a butterfly sitting in the wide open vertebral trough. The anterior horns, corresponding to the hind wings of the butterfly, were well outlined while the lateral and posterior horns formed cell masses extending in dorsolateral direction like the forewings (Fig. 9). Spinal ganglia lying near the base of the vertebral trough were clearly connected by anterior and posterior nerve roots with the anterior horns and with the laterally displaced posterior horns. Proceeding caudad, the neural plate decreased in size, but all the cell masses described remained well defined throughout the thoracic and abdominal regions.

The adrenal glands were well developed. Below the level of the kidneys the ventral subarachnoid space became smaller so that the neural plate lay within the vertebral trough. At the same level, penetration of a hepatic lobe and of intestine through a defect of the ventral abdominal wall could be seen (Fig. 11).

In subsequent sections, although the neural plate did not close by folding of its lateral wings, the spinal nervous tissue assumed more and more the shape of a normal spinal cord. In the pelvic region the caudal spinal cord had an anterior and a posterior central canal. The cord in its most caudal parts became covered at first by *stratum corneum*, then by skin and subcutaneous tissue. In its most caudal portion the cord was encompassed by cartilaginous vertebral arches, thus assuming a fairly normal appearance.

Closely related to the young with craniorachischisis were those with exencephaly only. There were 4 of these among the young from mothers treated with methyl salicylate and 6 from mothers treated with sodium salicylate (Fig. 7). These specimens differed in regard to size and shape of the exposed brain, but there was no rachischisis.

Four young had hydrocephalus, recognized on gross sectioning. One of these was examined histologically, and the aqueduct was found to be completely closed throughout the rostral two thirds of the midbrain but opened into the fourth ventricle in its caudal third. The ependymal lining of the aqueduct could be recognized throughout its closed portion. The lateral and the third ventricles were enlarged, but the fourth ventricle was of normal size.

Also probably related to these malformations of the central nervous system were anomalies of the axial skeleton seen in cleared specimens. As shown in Figure 6, there were anomalies of vertebrae and ribs, more or less expressed in the cervical, thoracic and lumbar regions. Only in two animals were the bones of the extremities grossly deformed.

Of a different type were 9 young that had harelip, oblique facial clefts and/or cleft palate. None of these young had craniorachischisis. There were 3 specimens, all from a mother treated with 0.4 cc. of methyl salicylate on the tenth day of pregnancy, which were of special interest. One of these young, illustrated in Figure 12, showed marked separation of the facial processes. The condition appeared as an exaggeration of the facial clefts seen in early embryonic stages. The maxillary processes were widely separated from each other and from the nasomedial processes, thus establishing a huge cleft of the upper lip. The maxillary processes were also separated from the nasolateral processes, maintaining a wide naso-optic fissure and forming an oblique facial cleft. The eyes were located in the angle between the maxillary and nasolateral processes. There were wide palatal clefts, but the mandibular processes were united.

Two of these unusual specimens were examined histologically, and many anomalies were found in the face, the eyes and the brain, but caudal to the thalamic area the animal appeared anatomically normal. The eyes were not covered by lids and presented histologic features which we had not observed previously in eyes rendered abnormal by other teratogenic methods. The external leaf of the retina was differentiated like the internal leaf, into nuclear and nerve fiber layers, but the layers of the external leaf were turned inside out. Thus the lens, iris and retina were enclosed in an outer shell of optic retina which was covered externally by a more or less preserved nerve fiber layer (Fig. 13). This paradoxical pattern can be explained by abnormal folding of the retinal leaves seen in other sections and interpreted as an extreme form of the eversion of the retina occurring in colobomas. In addition there were many anomalies of the cornea, iris, ciliary body and lens which will not be described in detail here.

Over the posterior portions of the nasal cavities, where the olfactory bulb appears normally, large masses of olfactory nerve were interposed between the pallium and the cranial base. In posterior sections the large rhinencephalon formed a broad base for the small, triangular pallium. The top of the pallium extruded between a gap of the cranial bones forming an anterior exencephaly, which, however, remained covered by skin, fibrous tissue or *stratum corneum* throughout, so that

brain tissue did not reach the surface of the head. In sections posterior to the foramens of Monro, the brain assumed normal shape and re-entered the cranial cavity.

Discussion

According to our present knowledge, salicylate poisoning is not one of the most efficient teratogenic methods available. This seems to be so because teratogenic doses of salicylates are close to doses lethal to the embryo and toxic to the mother, making the yield of young with congenital malformations relatively small. It should be emphasized that the doses employed always were, on a weight basis, far greater than therapeutic doses used in man.

Of the anomalies produced with some regularity, craniorachischisis is of particular interest. Anencephaly is one of the most frequent lethal malformations of the central nervous system encountered in the newborn period, and about 10 per cent of the cases are combined with *spina bifida*.[3] Although complete rachischisis (holorachischisis) is relatively rare in infants, it is of importance because its origin is closely related to the more common malformations of the central nervous system, such as anencephaly, exencephaly, and *spina bifida*. In human holorachischisis the vertebral column forms a flat trough, lined by a reddish, smooth, fibrous membrane, a derivative of undifferentiated meningeal tissue. There may be no spinal nervous tissue (amyelia), but spinal ganglia and nerves or nerve roots can be found on or in the meningeal lining. In other cases an *area medullo-vasculosa,* consisting of more or less preserved nervous and angioma-like tissue, may cover the vertebral trough. The skin defect is usually wider than the vertebral area so that the cutaneous cover of the trunk begins lateral to the rudiments of the neural arches.[4–7] Association with eventration is not rare.[5]

The resemblance of these human monsters to those induced experimentally is illustrated in Figures 1 and 2. The morphogenesis of craniorachischisis cannot be studied well in man, since the bulk of the central nervous system has usually deteriorated by the time of birth. But rat fetuses can be obtained with a well preserved, though exposed, central nervous system because the mother can be sacrificed before term, and destruction of the nervous tissue by the birth act prevented. In addition, the period of fetal growth following organogenesis is absolutely and relatively much shorter in the rat than in man so that intra-uterine deterioration is less marked in the abnormal animal fetuses. While the pathogenesis of craniorachischisis in man has to be reconstructed from the sparse remnants of the brain and

spinal nervous tissue found in the open skeletal trough, it can be observed directly in experimental animals.

In this preliminary study a few interesting facts have come to light. The exencephaly described in the animal with craniorachischisis consisted of a brain that was completely exposed with the exception of the olfactory lobes. If the brain had undergone destruction, anencephaly would have resulted. Although this brain showed many anomalies, its principal structure was sufficiently normal to indicate that the anterior portion of the neural plate had closed. It is usually assumed that the *area cerebro-vasculosa* of anencephalic monsters was preceded by a rostrally open medullary plate, and there is evidence from human[8] and animal embryos[9,10] that extroversion of the hemispheres can occur in such exposed brains. However, it has also been stated that in some cases the *area cerebro-vasculosa* may develop from a brain derived from a rostrally closed neural tube.[6] That this opinion is correct is suggested by the animal described. It seems that the rostral portion of the neural plate had closed normally, but that it had remained open in those portions from which the hindbrain, the medulla, and the major part of the spinal cord are formed. The most caudal part of the central nervous system was a spinal cord with a ventral and a dorsal central canal. Similar observations have been made in children with craniorachischisis, who often show a closed spinal column and cord in the terminal portion of the spine.[5] This is of considerable interest since this portion is derived from the tail bud and not formed by folding of the neural tube.[11]

In young rats with craniorachischisis the vertebral trough is covered dorsally by a well developed medullary plate which appears in cross section about twice as large as the spinal cord of a normal rat of comparable size. Overgrowth of abnormal neural tissue was also found by Patten[12] in human embryos. Figure 9 shows the precarious situation of the neural plate lying on the back of the animal. It is only loosely connected with the rest of the trunk by a thin layer of cornified epithelium and a few blood vessels and nerves. It is likely that this plate, which is exposed to amniotic fluid and to trauma, would be separated from the body and destroyed if normal termination of pregnancy were permitted. In animals with myeloschisis limited to the lumbar area, the onset of degenerative processes and shedding of the neural plate can be clearly recognized toward the end of gestation.[13] If one removed, in thought, the neural plate (Fig. 9), there would remain the vertebral trough covered by meningeal tissue containing spinal ganglia and nerves. This picture would resemble closely the state of craniorachischisis encountered in infants.[5,6] It is possible that

such lesions experimentally produced correspond to early stages of human fetuses with this anomaly, but it should be pointed out that in malformations of this nature, variations of morphologic details may occur between and within the species.

Only a few remarks will be made about the unusual specimens with extensive facial clefts (Fig. 12) and alterations limited to the most anterior portion of the central nervous system. Similar "fringe anomalies" are also obtained by other teratogenic methods. While some malformations are induced with regularity by a specific procedure, others occur only rarely "at the fringe of the pattern," and it is not possible to reproduce them at will. One wonders in such cases whether the abnormal pattern is brought about by the experiment or whether one is dealing with spontaneous malformations. The latter possibility seems unlikely if in many thousands of control rats such serious malformations have not been observed. It is of interest that oblique clefts and ocular malformations similar to those described above have been produced in two laboratories by pteroylglutamic acid deficiency.[14,15] It seems that with this method, oblique facial clefts are also only occasionally obtained.[15]

Summary

Congenital malformations can be induced in rats by salicylate poisoning of the mother while the embryos are in early stages of development. Among the anomalies produced was craniorachischisis with well preserved cerebral and spinal nervous tissues. It is suggested that the condition observed may be comparable to early stages of craniorachischisis in human fetuses. Other malformations encountered were exencephaly, hydrocephaly, facial clefts, eye defects, gastroschisis, and irregularities of the vertebrae and ribs.

REFERENCES

1. Warkany, J.; Kalter, H., and Geiger, J. F. Experimental teratology. With special reference to congenital malformations of the central nervous system. *Ped. Clin. N. America,* 1957, Nov., 983–994.

2. Dawson, A. B. A note on the staining of the skeleton of cleared specimens with alizarin red S. *Stain Technol.,* 1926, 1, 123–124.

3. Record, R. G., and McKeown, T. Congenital malformations of the central nervous system. I. A survey of 930 cases. *Brit. J. Social Med.,* 1949, 3, 183–219.

4. Ballantyne, J. W. Manual of Antenatal Pathology and Hygiene. The Embryo. W. Green & Sons, Edinburgh, 1904, 697 pp.

5. Kermauner, F. Missbildungen des Rumpfes. In: Die Morphologie der Missbildungen des Menschen und der Tier. By Schwalbe, E. G. Fischer, Jena, 1909, Vol. 3, Part 1, pp. 86–112.

6. Ernst, P. Missbildungen des Nervensystems. In: Die Morphologie der Missbildungen des Menschen und der Tier. By Schwalbe, E. G. Fischer, Jena, 1909, Vol. 3, Part 2, pp. 67–252.

7. Potter, E. Pathology of the Fetus and the Newborn. The Year Book Publishers, Inc., Chicago, 1952, 574 pp.

8. Hunter, R. H. Extroversion of the cerebral hemispheres in a human embryo. *J. Anat.*, 1934–1935, **69**, 82–85.

9. Bonnevie, K. Pseudencephalie als spontane recessive (?) Mutation bei der Hausmaus. *Skrifter utgitt av Det Norske Videnskaps—Akademi I Oslo. I. Matematisk-naturvidenskapelig klasse*, **2**, 1936, No. 9, 1–39.

10. Giroud, A., and Martinet, M. Morphogenèse de l'anencéphalie. *Arch. anat. Micr., Paris*, 1957, **46**, 247–264.

11. Arey, L. B. Developmental Anatomy. A Textbook and Laboratory Manual of Embryology. W. B. Saunders Co., Philadelphia, 1954, ed. 6, 680 pp.

12. Patten, B. M. Overgrowth of the neural tube in young human embryos. *Anat. Rec.*, 1952, **113**, 381–393.

13. Warkany, J.; Wilson, J. G., and Geiger, J. F. Myeloschisis and myelomeningocele produced experimentally in the rat. *J. Comp. Neurol.*, 1958, **109**, 35–64.

14. Nelson, M. M.; Wright, H. V.; Asling, C. W., and Evans, H. M. Multiple congenital abnormalities resulting from transitory deficiency of pteroylglutamic acid during gestation in the rat. *J. Nutrition*, 1955, **56**, 349–369.

15. Giroud, A. Les malformations congénitales et leurs causes. *Biol. méd.*, 1955, **44**, 524–609.

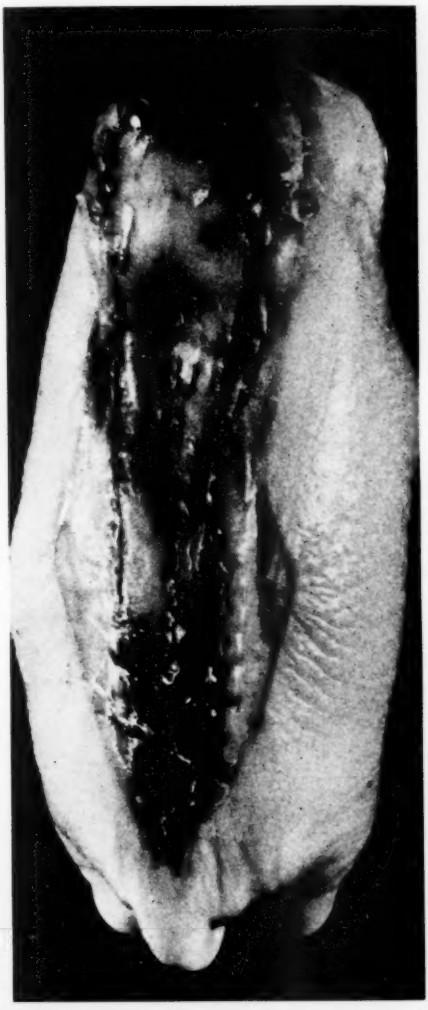
LEGENDS FOR FIGURES

Photomicrographs have been made of sections stained with hematoxylin and eosin.

FIG. 1. Craniorachischisis in a child. (From: Potter, E. L. Pathology of the Fetus and the Newborn,[7] Fig. 439B, p. 415. Used with permission of the author and publisher.)

FIG. 2. Craniorachischisis in a 21-day rat fetus. × 5.

FIG. 3. Six 21-day rat fetuses with craniorachischisis, showing exposed brains and neural plates. × 1.5.



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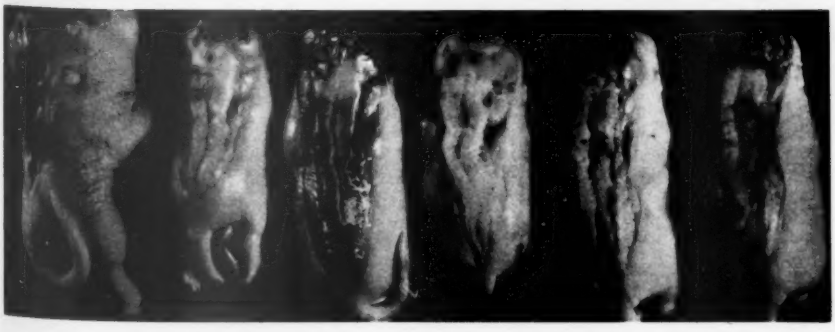

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FIG. 4. Frontal section through head of normal 21-day rat fetus. Optic nerves are seen leaving the eyes; the pallium is located over the rhinencephalon. × 9.

FIG. 5. Section through the head of young rat with craniorachischisis. The brain area is comparable to that of Figure 4. Note that brain is leaving the skull and protruding beyond the cranial defect. Because of caudad displacement of the brain, the eyes are not seen in this section. × 8.5.

FIG. 6. Cleared specimen, young of female rat treated with methyl salicylate, showing malformed vertebrae and ribs. × 4.

FIG. 7. Exencephaly and shortening of maxilla and mandible in young of female rat treated with sodium salicylate. × 4.

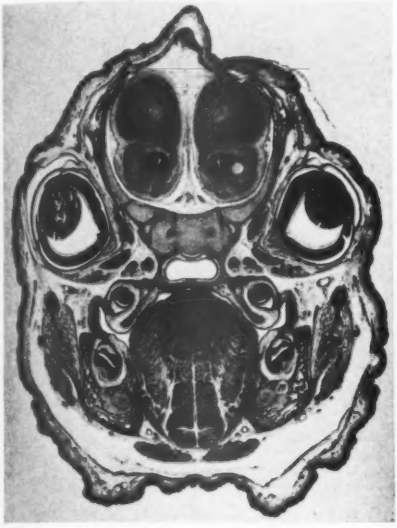

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FIG. 8. Frontal section through thalamus and hemispheres of young rat with craniorachischisis. The brain lies without osseous cover on the base of the skull. There is no extroversion of the brain, but many structural anomalies can be recognized. Note the triangular process in the sagittal fissure replacing the *tela chorioidea* of the third ventricle and pineal gland. × 20.

FIG. 9. Section through craniorachischisis, showing an open neural plate lying over the vertebral trough. × 30.

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FIG. 10. Fetus of female rat treated with methyl salicylate. Note craniorachischisis and eventration of liver and intestine. × 3.5.

FIG. 11. Section through the abdomen of young rat with craniorachischisis and eventration, showing protrusion of liver and intestine. × 8.

FIG. 12. Specimen with facial clefts. The maxillary processes are not fused with each other nor with the nasomedial and nasolateral processes. The left eye can be seen between the left maxillary and nasolateral processes. × 5.

FIG. 13. Frontal section through abnormal eye, showing external leaf of retina differentiated like internal leaf, with layers turned inside out. × 33.

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A PATHOLOGIC HUMAN EMBRYO*

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The discovery of a recently implanted human ovum in endometrial scrapings is unusual. If such an ovum should be abnormal, it will be of interest to those concerned with reproductive failure. This paper describes such a specimen and interprets it in the light of present knowledge.

The preformation theory that an embryo must exist in miniature in the egg was first questioned by Aristotle (384-322 B.C.) who suggested that differentiation may occur from a formless beginning.[1] Preformation continued to be widely accepted until the beginning of the 19th century, when the scientific approach had paved the way for the modern view that though development is strictly preformational as regards genes and hereditary influences, it is rigorously epigenetic in actual construction.[1]

Studies in early human embryology have recent origins, beginning with the work of His and Keibel in Europe and Minot and Mall in America.[1] *The Contributions to Embryology* of the Carnegie Institute of Washington, dating from 1915, contain many detailed descriptions of human embryos.

Hertig, Rock and Adams[2] referred to the lack of information still prevailing in 1934 on the first two weeks of human embryonic development. They described 34 recently implanted ova from 211 patients, each with known menstrual and coital dates preceding operation. It is significant that of these ova procured from patients of known high fertility with normal endometriums, 21 were morphologically normal, while 13 (slightly more than one third) were abnormal.

MATERIAL AND METHODS

The tissue examined consisted of endometrial scrapings from a woman of 29 years, who gave the following historical information:

27 March, 1950. Normal pregnancy, normal female child.
29 December, 1951. Normal pregnancy, normal male child.
25 September, 1953. Miscarriage of pregnancy at 3 months.
January, 1955. Normal birth. "Never really well since."
4 October, 1955. Moderate menstrual blood loss for 3 months.
11 October, 1955. Curettage. "Hyperplastic mid-intermenstrual phase endometrium."

* Received for publication, July 28, 1958.

7 October, 1956. Miscarriage of pregnancy at 2 months.
12 July, 1957. Menstruation.
25 August, 1957. Menstruation. "Often an interval of this length."
14 September, 1957. Acutely inflamed pelvic appendix removed.
16 September, 1957. Reported almost continuous bleeding *per vaginam* since onset of previous menses.
17 September, 1957. Curettage.
Dates of coitus were not obtained.

The specimen consisted of a few fragments and a larger ovoid piece of tissue measuring 10 by 7 by 3 mm., containing clear fluid. When its nature was appreciated, it was sectioned serially. Apparently the larger fragment, containing a tiny chorionic vesicle, was cut so that the embryonic disc, except for the original section, was lost in trimming; subsequent serial sections proceeded outwards from the disc edge. Measurements of the embryo were made by placing a slide micrometer in direct apposition with the section.

DESCRIPTION OF SPECIMEN

The Endometrium

The small amount of endometrium, in keeping with the preceding menstrual loss, consisted of basal mucosa and included fibromuscular tissue with a hyalinized spiral arteriole of the type seen in subinvolution of the uterus (Fig. 1). The superficial mucosa, though showing secretory activity, exhibited neither the late secretory endometrium nor the decidual alteration which would be expected in pregnancy (Fig. 2). At the implantation site, however, there was significant decidual change, but with degeneration and leukocytic infiltration (Figs. 3 and 4).

The Chorionic Vesicle

The Trophoblast. The trophoblast occupied a zone of hemorrhage around the vesicle, which appeared wholly embedded in the decidua (Fig. 3). It consisted of mixed immature, hydropic, and fibrotic chorionic villi and small, occasionally vacuolated clumps of syncytiotrophoblast. The vesicle, lying in maternal blood, had to some extent lost contact with the surrounding decidua. The latter contained only occasional trophoblastic wandering cells, although fibrotic villi were evident at some distance from the vesicle itself (Fig. 4).

The villi varied greatly in size, and though more or less evenly distributed over the vesicle (Fig. 3), were absent from some regions. The villous stroma was either fibrotic and hyalinized or showed early hydatidiform swelling (Fig. 5). The trophoblast lacked the florid fea-

tures of normal immature placental tissue and exhibited syncytial knot formation (Fig. 6). Most of the syncytiotrophoblast lay peripherally in the zone of hemorrhage, occasionally in contact with the surrounding decidua. Small vessels, containing primitive blood cells, were common (Fig. 7) but were absent from the smallest and the hydropic villi.

The average external diameter of the chorionic vesicle and trophoblastic zone was 4.25 mm.

The Extra-embryonic Cavity. This cavity was ovoid (Fig. 3), had an average diameter of 3.7 mm. and contained finely granular eosinophilic precipitate, blood cells and macrophages; some cells appeared to have "spilled" there during sectioning. The cavity wall (Fig. 6) consisted of a thin layer of moderately cellular mesoblast, applied to the chorionic membrane and continuous with the stroma of the protruding villi. In the wall of the cavity, as in the villi, occasional vessels contained nucleated erythrocytes and other blood cells (Fig. 6).

The Embryo (Figs. 3 and 8). The embryo lay centrally within the vesicle and consisted of the yolk sac and the larger, ruptured, bilaminar amniotic sac (approximate diameter, 1.2 mm.) continuous with, and partially enclosing, the developing embryonic disc. The body stalk probably was partly represented by a finger-like projection from the junction of the embryonic mass with the yolk sac.

The yolk sac (Fig. 8) was an ovoid vesicle (average diameter, 0.35 mm.) attached to the ventral surface of the embryonic mass. Like the amniotic sac, it had a thin, relatively acellular wall lined on each surface by flattened cells. Its cavity contained cellular, granular precipitate similar to that in the extra-embryonic cavity. Dense foci of hemopoiesis occurred in the yolk sac wall, from the outer aspects of which delicate vessels sprouted into the extra-embryonic cavity.

The embryonic mass (Fig. 8) was an asymmetrical collection of cellular tissue with an average diameter of 0.37 mm. Its most ventral portion was continuous on each side with the amniotic sac. The lateral extensions differed greatly in shape, thickness, and cellularity. The embryonic mass projected into the amniotic cavity and contained 3 major structures.

The most conspicuous feature, just beneath the dorsal surface, was an eccentric rosette (0.1 mm. in diameter) with at least one dorsal spine composed of cells with pyknotic nuclei. It contained amorphous eosinophilic material and occasional nuclei, some showing disintegration. Its position and form suggested a neural tube; the asymmetrical dorsal spines could represent neural crests. Close beneath was a rounded space (0.15 mm. in diameter) containing a few nucleated

erythrocytes and neutrophils. It was lined by a single layer of flattened cells and appeared to be a large blood vessel, presumably the fused dorsal aortas. Again beneath, and lying medially in the most ventral portion of the mass, was a hollow ovoid structure (0.06 mm. in diameter) containing debris and lined by a layer of columnar cells with small dark nuclei; this was presumably either gut or allantois.

There was no clear indication of notochordal or somite development. Throughout, cell boundaries were not visible; nuclear pyknosis and cellular vacuolation were common; mitotic figures were not seen, and there was an extensive infiltration with leukocytes, often polymorphonuclear, many showing disintegration.

Attempts to define the "nuclear sex" of the embryo, utilizing the criteria of Barr, Bertram, and Lindsay,[3] were unsuccessful, probably because of the degenerative alterations described.

Discussion

Despite the limited observations, the rarity of this specimen, its abnormalities, and the growing interest in reproductive failure justify this description. Uncertainty as to the level and plane of the sections increases the difficulty in interpreting its details, but the features described are re-examined in an attempt to assess the age of the embryo and the significance of its abnormalities.

Scipiades[4] noted that methods of assessing embryologic age varied with different authors, some attaching greatest importance to embryonic structure while others emphasized chorionic differentiation. Before the development of the primitive streak, the chorionic component is more precocious, and after this the embryo provides more details for assessment. Since observations were confined to one section of this embryo, the applicability of this "yardstick" was limited, so that all aspects such as the maternal history, trophoblastic development, hemopoietic and angiogenic activity, embryonic and vesicle size, and descriptions of other human embryos must be considered.

Ingalls[5] quoted Mall[6] as stating that ova of the same fertilization age might show differences in size and development because of different environmental factors during early development, and that such disparities were most evident in abnormal ova. Ingalls described an embryo with the vesicle size commensurate with the assigned age, which was disproportionately large in regard to chorionic development and estimated age, and very large, considering its degree of development. He postulated a stimulus to embryonic growth and said that the reverse disproportion between sac and embryo was characteristic

of pathologic ova. He stressed the importance of environmental factors in rate and direction of development.

Bartelmez and Evans[7] reached similar conclusions; they asked whether the conditions producing abortion could not also modify the rate of embryonic development. They pointed out that in abortions there was no reliable information as to the time elapsing between cessation of active growth of the ovum and its expulsion from the uterus. Further, it is often assumed that fertilization follows insemination promptly and that the survival time of the sperm in the tube is brief. Because of such uncertainties, very few young ova are available whose related clinical histories merit implicit confidence in a scientific discussion. These views were shared by Arey[1] who noted the importance of determining the time of fertilization and the difficulty in achieving this from data usually supplied. Gruenwald[8] stressed the importance of necrotizing agents as causes of retrogression in previously normally developed embryos. Specimens affected in this way might give false impressions of embryonic youthfulness. These considerations indicate the speculative nature of any attempt to assess the age of this embryo.

It is appropriate first to examine the maternal history from which the possible extremes of conceptual age may be derived. The significant menstruation occurred on July 12, 1957, and was followed 44 days later (an interval not uncommon with this patient), on August 25, 1957, by a further blood loss which continued thereafter until the termination of pregnancy on September 17, 1957, a total period of 67 days.

In the absence of fertilization dates, assessment of conceptual age begins with an estimation of the time of ovulation. It has long been accepted that ovulation occurs normally 15 days before the onset of the next expected menstrual period,[9] and that this applies to cycles of whatever length. Applying this generalization to the temporary assumption that July 12, 1957, marked the onset of a normal cycle of about 28 days, we would conclude that ovulation and fertilization occurred on or about July 26. If, however, July 12 marked the onset of a 44-day cycle, we might conclude that ovulation and fertilization occurred on or about August 10. Also it may be assumed either that the menstruation beginning on August 25 indicated failure of the pregnancy or that it represented a normal menstrual loss (such as sometimes occurs early in pregnancy), later merging with bleeding due to failure.

From these considerations, 5 possible, approximate, conceptual

ages emerge as follows:

July 26 to September 17: 53 days (maximum possible age)
July 26 to August 25+: 30+ days
July 26 to August 25: 30 days
August 10 to August 25+: 15+ days
August 10 to August 25: 15 days (minimum possible age)

Hertig and co-workers[2] have shown that vascular primordia appear in the villi and yolk sac wall of 13 to 16 day ova and coalesce to form a functional circulation by 21 to 23 days. That this embryo was certainly more than 16, and perhaps more than 21 days of age is suggested by the relatively mature chorionic villi (showing degenerative changes requiring some time to occur after villous formation), and by the relatively advanced angiogenesis. Moreover, this embryo showed a much greater development than those of 16 days illustrated by Hertig and associates[2] and is morphologically similar (Fig. 8) to a 24 somite human embryo of about 28 days recorded by Johnson[10] and illustrated in his Figure 9, Plate 8. These interpretations are compatible with standard accounts of embryologic development in the fourth week.

Thus, the neural groove begins to form the neural tube in embryos of about 6 somites[1] (at about 20 to 22 days[11]); this process extends cranially and caudally from the mid-zone at similar rates, the caudal extension usually being completed in the 25 somite stage[1] (at about 29 days of age[11]). By the 28th day, the dorsal aortas have fused throughout much of their lengths,[1,10] and the separation of the primitive gut from the yolk sac occurs between the third and fifth weeks.

The measurements cited were obtained after fixation, embedding and sectioning, and therefore differ from measurements obtained *in vivo*. Bartelmez and Evans[7] noted differences in yolk sac dimensions according to fixative and estimated shrinkage to vary from 10 to 36 per cent, depending largely on the time elapsing before fixation.

Table I is based entirely on such measurements and compares this embryo with some others described. It shows that by comparison with other normal specimens, the dimensions indicate an age of something less than 16 days. Its development, however, is so far in advance of normal specimens of this age that such a conclusion must be rejected. Its anomalous size is probably due to degenerative and retrogressive changes. Altogether, therefore, it would appear that the developmental age or period of progressive growth of this embryo lies in the vicinity of 30 days, beginning with fertilization on or about July 26.

It may appear astonishing that at the time of curettage more than

TABLE I

Comparison of the Measurements of the Present Specimen and Those of Other Embryos Described in the Literature

Author	Designation	Date	Est. Age (days)	Chorionic vesicle Av. diam. (mm.) External	Chorionic vesicle Av. diam. (mm.) Internal	Amniotic sac Av. diam. (mm.)	Yolk sac Av. diam. (mm.)	Embryonic structure and measurements (mm.)
Arey[1]	(U.C. 1496) Brewer	1938	14	2.8	1.5		0.2	L × W = 0.21 × 0.18. Beginning primitive streak.
Present specimen		1958		4.25	3.7	1.2	0.35	Thickness 0.37. See text.
Arey[1]	(Mateer) Streeter	1919	16	6.8	4.7		1.5	L × W = 0.92 × 0.78. Primitive groove: villi branching.
Arey[1]	Thompson-Brash	1923	17	7.2	5.7		0.9	L × W = 0.9 × 0.9. Solid head process. Cloacal membrane.
Arey[1]	(W.R. 1) Ingalls	1918	18	7.9	6.7		2.5	L × W = 2.0 × .08. Notochordal canal tunnelling head process.
Arey[1]	(Glae) Spee	1889 1896	19	9.3	7.8		2.1	L × W = 1.5 × 0.6. Neural folds. Neurenteric canal. Foregut indicated.
Hamilton *et al.*[11]	Ingalls	1918	21	6.3				2.00 × 0.75
Atwell[12]	C.C. 5072	1930	22(?)	10.3			3.0	Length 2.81. 17 somites
Corner[13]	C.C. 5074	1929	28(?)	15.0				10 somites
Heuser[14]	C.C. 4529	1930	28(?)	21.6				14 somites

3 weeks later the embryo was in such a relatively good state of preservation. Apparently the total life of the embryo exceeded its developmental age by a period of slow retrogressive alteration still operating at the time of curettage. These conclusions illustrate the difficulties involved in assessing embryonic age and support the observations of previous authors.

The Abnormalities of the Specimen

Although the vesicle appeared to be wholly embedded in decidua (Fig. 3), sections in other planes might have shown that this was not so. However, clumps of degenerated villi at some distance from the implantation site (Fig. 4) contrasted with the almost complete absence of trophoblastic cells beyond the zone of maternal hemorrhage and suggest that implantation was initially vigorous.

Hertig,[15] who kindly commented on one of the sections, noted the early but definite hydropic or hydatidiform swelling of the villous stroma (Fig. 5) and considered the whole to be a good example of the early blighting of a fertilized ovum. Thus, it would seem that implantation, though active initially, subsequently, because of factors unknown, became gradually less energetic. Similar circumstances apply in the case of a pathologic ovum in the Carnegie collection (no. 7205) described by Ramsey[16] who noted the poverty of trophoblast and villi and the extravasated maternal blood. She suggested that elaboration of syncytium and mesoderm had exhausted the parent cytotrophoblast and that "for some reason unknown, presumably an inherent defect in the constitution of the egg, proliferation of these parent cells, which should be continuous, ceased at an early stage when the villi were just beginning to form." Witschi[17] considered that such inherent defects or hereditary factors were sometimes responsible for "poor quality" human ova.

Although asymmetry in the early stages of normal development has been recorded,[7] it seems unlikely, for instance, that the difference between the two attachments of this embryo with the amniotic sac (Fig. 8) was normal.

Other apparent anomalies included the absence of extra-embryonic mesoblast on the outer aspects of the amniotic and yolk sacs and the absence of notochord and somites. However, variable degrees of development of exocoelomic mesoblast, not absolutely correlated with size and development of normal embryos, have been described.[2]

Maternal abnormalities included the involuting secretory endometrium and necrotic decidua and the history of previous abortions and

menstrual irregularity. These suggest a chronic maternal hormonal dysfunction, the importance of which has been stressed by many observers[5,6] and which may have been responsible for the previous abortions in the present patient.

Since retrogression appeared to have begun some 3 weeks before the appendectomy, it is unlikely that this operation, only 3 days before curettage, contributed significantly to the final pattern. Nevertheless, a much earlier, perhaps unrecognized inflammatory episode in that neighboring organ, or any one of a variety of "minor" infections, must be considered in view of the increasing recognition of their association with anomalous development.

However, Hertig,[15] who was unacquainted with the maternal history at the time he examined one of the serial sections, considered that the maternal bleeding might have been due to withdrawal of hormonal support secondary to a blighted ovum whose trophoblast was intrinsically defective and had been declining in vigor. He found similarities between this and younger specimens which he and his co-workers described.[2] They observed structurally normal endometrium in the presence of a wide spectrum of abnormalities in the ova and indications of lessened activity of the associated *corpora lutea*. From the progestational endometrial alterations, they concluded that the initial qualities of the *corpora lutea* were normal and that their failure to undergo secondary hyperplasia as in normal pregnancy was due to inadequate stimulation by a defective trophoblast.

There was no opportunity in this case to examine the associated *corpus luteum*.

Discussion

We have here a "failed" pregnancy, destined to abort. That this failure may have been due to maternal factors is supported by the the history suggesting a chronic hormonal dysfunction. That it may have been due to deficiencies intrinsic to the embryo cannot be denied, in which case genetic factors may be implicated. The increasingly popular view that a common cause of abortion is a genetically defective ovum or sperm, or both, is mirrored in the above interpretation of Hertig[15] and supported by other authors.[2,17–19]

Witschi[17] doubted whether as many as 20 per cent of all ovulations produced "good eggs" and quoted Hertig and Rock[20] as showing that, of ovulated eggs, more than half were either not fertilizable, or resulted in zygotes of such poor quality that they decomposed soon after impregnation, and that of all ova recovered during the first two weeks, about one half exhibited visible abnormality.

The evidence that genetic factors may condition developmental anomalies derives from the peculiar distributions of the anomalies concerned, and its elucidation presupposes examination of large portions of the family tree of the affected individuals. Therefore, although genetic interpretations are legitimate, they become more tenuous as such examinations become limited. To invoke this interpretation in single instances of pathologic ova occurring in women of proven high fertility is again legitimate but highly speculative and seems to disregard a considerable body of evidence implicating a wide variety of environmental factors in the causes of anomalous development and abortion.

It must also be recognized that genetic and environmental factors individually innocuous may in combination divert the normal course of pregnancy, and the possibility of such a combination must be considered in the present case. During the growth of knowledge of pathologic processes, many conditions once regarded as hereditary in origin have been found to have environmental causes. This was shown first in conditions in mature adults (i.e., tuberculosis and rickets) and more recently in those apparent at birth (i.e., malformations associated with rubella and exposure to roentgen rays).

It is highly probable that similar conclusions will emerge on the earliest phases of development. The idea that entirely different factors apply to the developing embryo seems to indicate the influence of the old embryologic hypothesis of preformation. The mechanism of failure in the present case is obscure and may be complex; a variety of environmental conditions may cause anomalous development, and although hormonal dysfunction has been suggested, other factors of a different kind are not thereby excluded.

The absence or inadequacy of examinations and records relating to aborted material limit our knowledge of the relationship between anomalous development and abortion. To date, the majority of descriptions of embryologic phenomena has concerned normal specimens. The small number of pathologic embryos described reflects our inexperience in this matter and highlights the need for care in handling potential specimens and for the further information certain to be revealed by their analysis.

Summary

A specimen of a pathologic fertilized human ovum contained in endometrial scrapings is described. It is concluded that embryonic "life" exceeded the developmental age (estimated at about 30 days) by a considerable period during which degenerative alterations oc-

curred. These changes and the uncertainty of the level of the sections obtained have contributed to difficulties of analysis.

The probable general importance of environmental factors in reproductive failure, suggested in this case by the maternal history, is contrasted with the growing use of genetic interpretations in such instances.

REFERENCES

1. Arey, L. B. Developmental Anatomy. W. B. Saunders & Co., Philadelphia and London, 1954, ed. 6, 680 pp.
2. Hertig, A. T.; Rock, J., and Adams, E. C. A description of 34 human ova within the first 17 days of development. *Am. J. Anat.*, 1956, **98**, 435–493.
3. Barr, M. L.; Bertram, L. F., and Lindsay, H. A. The morphology of the nerve cell nucleus, according to sex. *Anat. Rec.*, 1950, **107**, 283–297.
4. Scipiades, E., Jr. Young human ovum detected in uterine scraping. *Contrib. Embryol.*, 1938, **27**, 95–105.
5. Ingalls, N. W. A human embryo before the appearance of the myotomes. *Contrib. Embryol.*, 1918, **7**, 111–134.
6. Mall, F. P. On stages in the development of human embryos from 2 to 25 mm. long. *Anat. Anz.*, 1914, **46**, 78–84. Cited by Ingalls.[5]
7. Bartelmez, G. W., and Evans, H. M. Development of the human embryo during the period of somite formation; including embryos with 2 to 16 pairs of somites. *Contrib. Embryol.*, 1926, **17**, 1–67.
8. Gruenwald, P. Malformations caused by necrosis in the embryo. Illustrated by the effect of selenium compounds on chick embryos. *Am. J. Path.*, 1958, **34**, 77–103.
9. Knaus, H. H. Über den Zeitpunkt der Konzeptionsfähigkeit des Weibes. *Arch. f. Gynäk.*, 1931, **146**, 343–357; and *München med. Wchnschr.*, 1931, **78**, 344–347. Cited by Reynolds, S. R. M. Physiology of the Uterus. Paul B. Hoeber, Inc., New York, 1949, ed. 2, p. 36.
10. Johnson, F. P. A human embryo of 24 pairs of somites. *Contrib. Embryol.*, 1917, **6**, 125–168.
11. Hamilton, W. J.; Boyd, J. D., and Mossman, H. W. Human Embryology. (Prenatal Development of Form and Function.) W. Heffer & Sons, Ltd., Cambridge, 1945, pp. 34, 88.
12. Atwell, W. J. A human embryo with 17 pairs of somites. *Contrib. Embryol.*, 1930, **21**, 1–24.
13. Corner, G. W. A well-preserved human embryo of 10 somites. *Contrib. Embryol.*, 1929, **20**, 81–99.
14. Heuser, C. H. A human embryo with 14 pairs of somites. *Contrib. Embryol.*, 1930, **22**, 135–154.
15. Hertig, A. T. Personal communication, 1957.
16. Ramsey, E. M. The Yale embryo. *Contrib. Embryol.*, 1938, **27**, 67–84.
17. Witschi, E. Overripeness of the egg as a cause of twinning and teratogenesis: a review. *Cancer Res.*, 1952, **12**, 763–786.
18. Shettles, L. B. The ovum in infertility, abortion, and developmental anomaly. *Fertil. & Steril.*, 1956, **7**, 561–572.
19. Novak, E. Cited by Shettles.[18]

20. Hertig, A. T., and Rock, J. Abortive Human Ova and Associated Endometria. In: Menstruation and Its Disorders. Engle, E. T. (ed.). Proceedings of the Conference Held Under Auspices of the National Committee on Maternal Health. Charles C Thomas, Springfield, 1950, pp. 96–137. Cited by Witschi.[17]

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LEGENDS FOR FIGURES

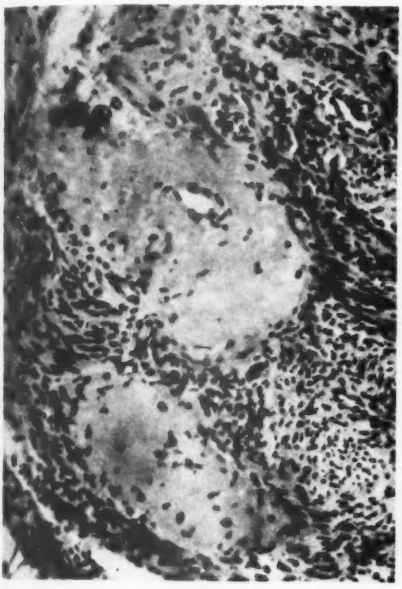
All sections illustrated were stained with hematoxylin and eosin.

FIG. 1. A fragment of basal endometrium, showing the fibromuscular tissue of the myometrium surrounding two portions of a thick, hyalinized, spiral arteriole of the type often seen in subinvolution of the uterus. × 165.

FIG. 2. A fragment of superficial endometrium, showing a secretory glandular pattern; the stroma, though edematous, is not decidual in appearance. × 65.

FIG. 3. The original section, showing the inflamed decidual fragment in which the chorionic vesicle appears to be completely embedded. The vesicle is surrounded by a narrow zone of maternal hemorrhage in which lie the chorionic villi and clumps of syncytiotrophoblast. Within the vesicle is the developing embryo projecting into the ruptured amniotic sac (on the left). The smaller yolk sac is on the right. × 14.

FIG. 4. The decidual fragment, showing widespread inflammatory infiltration around a small cluster of fibrotic chorionic villi found at some distance from the implantation site itself. × 100.



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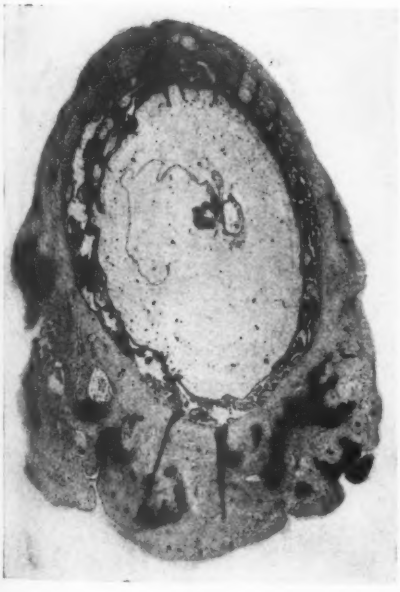

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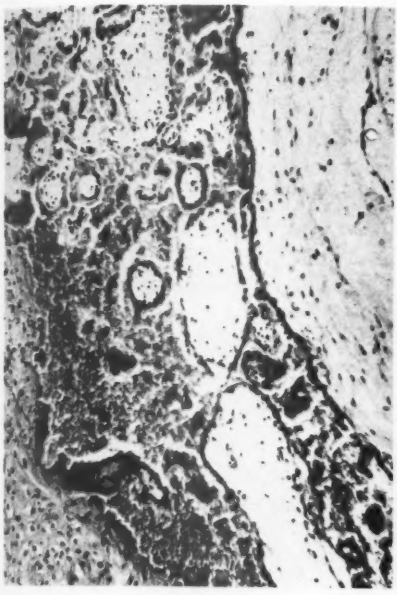
FIG. 5. A portion of the chorionic vesicle is shown on the upper right. The variety in size of its villi, and the hydropic nature of the largest of these can be seen. Clumps of syncytiotrophoblast are seen within the zone of hemorrhage and along the surface of the inflamed decidua (lower left). × 65.

FIG. 6. A fibrotic chorionic villus with syncytial knots (left). On the right is delicate mesoblastic tissue lining the inner surface of the chorionic membrane (center). Within it are two well defined blood vessels containing nucleated erythrocytes and other blood cells. × 265.

FIG. 7. A chorionic villus lying within the dark zone of maternal hemorrhage. Within its mesoblastic stroma are small but well defined thin-walled blood vessels (upper left and also center right) containing primitive blood cells, nucleated erythrocytes and various leukocytes. × 265.

FIG. 8. The original section, showing the developing embryonic disc (above) with the bilaminar amniotic sac attached to its lateral margins. The yolk sac containing blood cells lies beneath. Its wall contains a hemopoietic focus and shows angiogenic activity (lower left). Note the cellular and structural asymmetry of the embryonic mass, especially at the two points of junction with the amniotic sac. Within the mass are the 3 anatomic structures described in the text. × 100.



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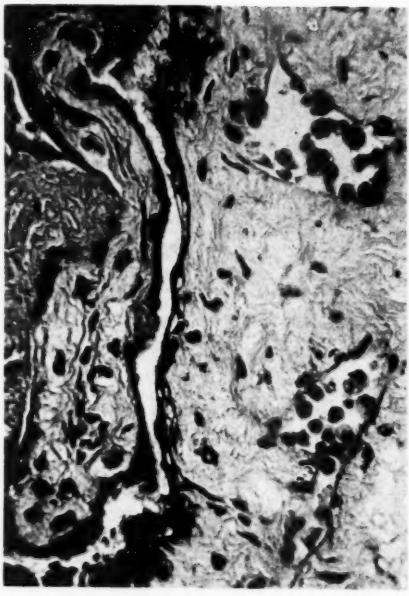

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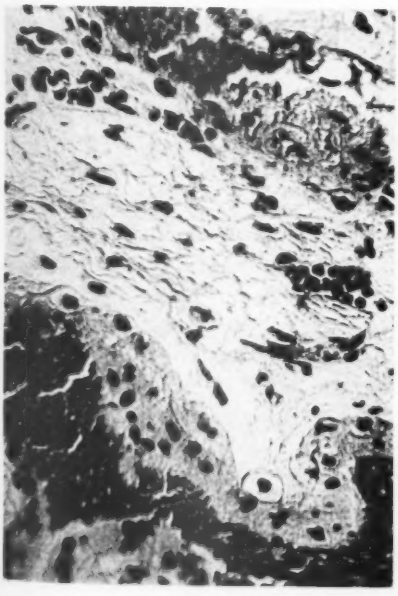

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EXPERIMENTAL DIETARY SIDEROSIS*

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The etiology and pathogenesis of siderosis, so widespread and frequently so severe among Africans in the Union, is still a subject of considerable controversy.[1-4] Especially debated are the significance of malnutrition, of dietary iron overload in the production of this interesting pathologic feature and, especially, of other possible factors permitting or actively promoting excessive iron absorption. Answers to these questions may throw considerable light not only on dietary siderosis in human subjects but on many aspects of iron metabolism in general.

Two major schools of thought have arisen in relation to the etiology of African siderosis. The one maintains that dietary iron overload, due to the reported high iron content of the African diet, is alone responsible for the excessive iron uptake and its subsequent deposition in the tissues.[3] According to those who hold this view, there is no need to invoke malnutrition or any nutritionally induced or other cellular metabolic disorder to account for the phenomenon. The other school of thought, while always maintaining that the excessive tissue iron is derived from the diet,[2] considers that the excessive absorption of dietary iron is an expression of some primary underlying disorder of cellular metabolism, perhaps consequent on long-standing and probably intermittent chronic malnutrition.

It has now been shown that the so-called "mucosal block" to iron uptake varies and, in nonanemic individuals, can be overcome at least by dietary iron overload. Thus, the feeding of large quantities of iron increases the total uptake of this mineral although the proportion of iron absorbed decreases progressively with the increase in the dose administered. This has now been repeatedly confirmed for acute experiments in man and animals.[5-8] It has also been shown experimentally that greater quantities of iron are absorbed and deposited in the tissues even if a relatively good diet is enriched with iron.[6,8] The investigators of the latter have, however, clearly demonstrated that greater amounts of iron accumulate in the tissues of rats fed an inadequate but iron-enriched diet than in animals given the same amounts of iron in a "good" diet. Their experiments thus indicate that the proportion

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of iron absorbed is in some way a function of the non-ferric constituents of the diet. Hegsted, Finch and Kinney[9] revealed that even on a "bad" (maize) diet, iron uptake is in part a function of the dietary phosphate content; however, they found that the concentration of several amino acids in the diet may also affect iron uptake, independently of the phosphate content. More recently, too, several investigators have shown that the calcium, vitamin C and vitamin E contents of the diet influence not only the rate of iron uptake but also the speed of its incorporation into hemoglobin, i.e., the intermediary metabolism and utilization of iron.[10]

It seemed important, therefore, to study further the effects of dietary iron overload and of malnutrition on iron uptake and tissue siderosis. This was all the more necessary in view of the absence from the literature of any data concerning the histogenesis of nutritionally induced siderosis and especially about the way excessively absorbed dietary iron is deposited in the tissues in the early stages of such experiments.

The object of the present study is to assess the effects of feeding excessive quantities of iron to rats maintained on a "good" as opposed to a "bad" diet, with a view to determining whether properties of the diet other than its iron content would influence the amount and distribution of iron in the body. Particular attention has here been paid to the hepatic changes at different stages of these experiments and to the site of initial appearance of histochemically demonstrable iron, first in the liver but later also in other tissues. Comparisons have also been made between the histochemically and chemically demonstrable iron content of various tissues.

Material and Methods

Male Wistar-strain rats initially weighing 200 to 230 gm. (mean weight: 215 gm.) were divided into 4 groups and fed *ad libitum* on our stock diet or uncooked mealie-meal (corn meal), with or without supplements of 4 per cent ferric citrate. The iron, calcium and phosphorus contents of these 4 diets are shown in Table I. In addition, all groups of rats were fed milk 3 times weekly and fresh vegetables twice weekly. At 50 and 100 days respectively, half the rats in each group were subjected to laparotomy and wedge-shaped specimens removed from the livers. The biopsy tissue was fixed in 10 per cent neutral formalin, and paraffin-embedded sections were stained with hematoxylin and eosin and by the ferrocyanide method for iron.[11]

At the conclusion of the experiment at 156 days, the rats were anesthetised with ether, and were bled by cardiac puncture for iron

estimations. The rats were then perfused with warm iron-free saline. Weighed specimens from various organs were removed for histologic examination and for iron estimations. The remaining carcasses were minced and also analyzed chemically for iron.

The iron contents of the various organs and carcass were determined by the α-α-dipyridyl reagent after wet digestion with sulphuric

TABLE I
Summary of Chemical Observations

	Group I	Group II	Group III	Group IV
Diet	Stock	Mealie meal	Stock + iron	Mealie meal + iron
Number of rats	5	5	10	7
Mean daily food intake per rat (gm.)	23	13	20	11
Dietary minerals (mg./100 gm. dry weight)				
Iron	77	4.2	1017	974
Phosphorus	830	6.7	818	61
Calcium	2640	33	2584	29
Mean daily iron intake per rat* (mg.)	18	0.6	203	107
Mean daily iron absorption per rat* (mg.)	0.19	0.26	0.35	0.57
Mean weight change per rat during experiment (gm.)	+140	+115	+87	−17
Concentration of iron (mg./100 gm. wet weight)				
Total body	8.2±0.5†	12.3±0.6	18.5±1.0	45.1±2.2
Liver	32.3±2.5	22.1±2.4	66.6±6.3	367±19

* Calculated from food and milk consumed in each group.
† Standard error of the mean.

and perchloric acids. Total body iron was calculated by adding, to the determined carcass iron, estimates of (a) the small samples of tissue removed for histologic and chemical study, and (b) total hemoglobin and plasma iron.

OBSERVATIONS

Growth. Increase in the body weight was greatest in animals fed the stock diet, slightly less in rats on mealie-meal alone, less still in those consuming the iron-enriched stock diet, and least of all in the animals fed mealie-meal supplemented with iron. It is clear from Table I that the addition of large quantities of iron suppressed the growth of rats consuming either the stock or the mealie-meal diets.

Histologic Observations

Liver. Even after 50 days there was a striking amount of histochemically demonstrable iron in the livers of rats fed the iron-enriched mealie-meal diet. At this stage, the iron-containing pigments were almost entirely confined to the liver cells in the portal third of each lobule. Only minimal amounts of stainable iron were detectable in the sinusoidal or portal phagocytes (Figs. 1 and 2). The affected liver cells were somewhat vacuolated and their walls were prominent ("plant-like"), the appearances resembling those previously described in the liver cells of man in "anoxia."[2,12] At this time iron was not demonstrable histochemically in the livers of any of the other groups of rats.

By the 100th day of the experiment, the alterations ascribed to anoxia and the hepatocellular siderosis in the rats fed the iron-enriched mealie-meal diet were even more marked in the liver biopsy specimens obtained at laparotomy. This is well exemplified in Figure 3, in which, apart from the alterations in the hepatic cells, the Kupffer cells and even some portal tract phagocytes can be seen to be laden with iron pigment. A typical example of the degree and extent of hepatocellular and phagocyte siderosis, at the end of the experiment (156 days) in rats fed mealie-meal and iron, is shown in Figures 4 and 5. Especially noteworthy is the greater extent of the siderosis of the liver cells in each lobule. In liver cells near the central veins, iron appeared as fine granules localized mainly along the biliary poles (Figs. 7 and 8) in a manner closely resembling that previously described in the early stages of siderosis in Africans.[1,2,13,14] In the periportal liver cells, however, iron-containing pigment occurred in coarser clumps scattered throughout the cytoplasm of the affected cells (Figs. 9 and 10). This appearance is indistinguishable from the Type III stage of siderosis described and portrayed in siderotic livers of Africans.[1,2,13] The hepatic phagocytes were also more heavily laden with iron pigment and many clumps of siderotic sinusoidal and portal phagocytes were now regularly observed (Figs. 4 and 5).

In contrast with the livers of rats fed mealie-meal and iron, the livers of animals fed the iron-enriched stock diet contained only minimal amounts of histochemically demonstrable iron, and this was confined to a few sinusoidal and portal phagocytes (Fig. 6) even though, as will be shown, the concentration of chemically-estimated hepatic iron at this stage doubled itself as compared with rats fed the stock diet alone for the same period of time.

Other Organs. Iron was not detected microscopically in either the

parenchymal or the connective tissue cells of the pancreas, heart, stomach and other tissues examined from any of the groups enumerated above. The splenic tissue of rats fed either the stock or the mealie-meal diets enriched with iron did appear, histologically, to be more heavily siderotic, the spleens of the mealie-meal plus iron group being perhaps slightly more laden with iron, but not very strikingly so (compare Figs. 11 and 12). The same remarks apply to the bone marrow (Figs. 13 and 14.). In the rats fed the stock diet plus iron, both the numbers and the sizes of the iron-laden phagocytes in the bone marrow were less than in those fed mealie-meal plus iron. This can be readily appreciated by studying any randomly selected field in the photomicrographs of bone marrow.

Very fine granules of iron were demonstrable in scattered epithelial cells of the proximal convoluted renal tubules of rats fed mealie-meal plus iron. This was not the case in the kidneys of the other groups.

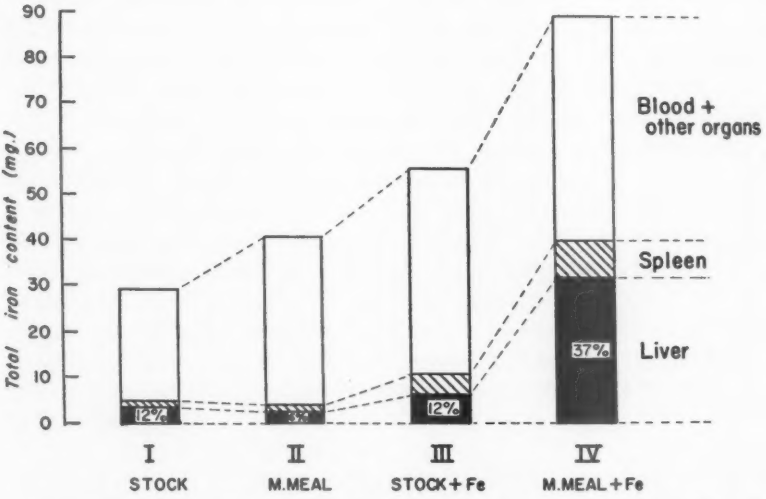
Especially striking were the differences in the iron content of the colonic surface epithelium, *tunica propria* and submucosa in rats fed mealie-meal and iron as compared with those given the iron-enriched stock diet for 156 days (compare Figs. 15 and 16). In the former group, the lumen poles of almost all the surface epithelial cells revealed numerous fine iron-containing granules closely resembling the lesions described by Gillman and Ivy[15] in the duodenal mucosa of iron-fed guinea pigs. Only occasional granules of iron were detected in the colonic surface cells of the control group (i.e., stock diet plus iron). Even more striking, however, were the massive accumulations of iron in the phagocytes of the *tunica propria* of the rats fed mealie-meal plus iron, when they were examined at the end of the experiment. It is generally considered that the duodenum and jejunum play a most active role in iron absorption; certainly in the guinea pig and in man, this is where iron is most commonly encountered in both the surface epithelium and phagocytes of the *tunica propria*.[2,15] However, only minimal amounts of epithelial and phagocyte iron were seen in the duodenum even of the heavily siderotic rats fed mealie-meal and iron (Fig. 17).

Whether the colonic iron, here described and portrayed, is being absorbed or excreted cannot be stated without the aid of radioactive tracers. It should be noted, however, that Granick[16] did describe a moderate increase in the ferritin demonstrable in the colonic mucosa of guinea pigs some time after a single dose of orally administered iron. The latter evidence suggests that the colon can absorb some dietary iron, at least in the guinea pig.

Chemical Data

A summary of the results of the chemically estimated iron content of the entire rats and of various tissues, studied at the end of the experiments, is presented in Table I.

The following are the most striking observations: (1) The concentration of total body iron was increased in the groups fed excessive quantities of iron added either to the stock or to the mealie-meal diets. In the former, the total body iron concentration rose to just over twice that of animals fed only the stock diet. In the rats which consumed mealie-meal plus iron, however, the total body iron concentration rose to almost 4 times that of the mealie-meal controls and to almost 2½ times that found in the group fed the iron-enriched stock diet. Comparisons of total iron content of different organs are shown in Text-figure 1.



Text-figure 1. Mean total iron content of liver, spleen and whole animal after 156 days on various diets. Percentages denote contribution of hepatic iron to total body iron in each group.

(2) From Table I and Text-figure 1 it can be seen that the increase in the concentration of total body iron, most notable in groups III and IV, was found to be associated with a two-fold increase in hepatic iron concentration in group III (compared with group I) while in group IV rats the hepatic iron concentration increased by 16-fold as compared with group II. In fact, the concentration of iron in the livers of group IV rats was almost 6 times greater than that in the livers in group III rats. In the rats fed mealie-meal plus iron (group IV), the

livers contained over 30 per cent of the total body iron as compared with 2.4 to 6.5 per cent of the body iron in the other groups (Text-fig. 1). This indicates that rats fed the iron-enriched mealie-meal diet not only absorbed and retained far more iron than any other rats, but in addition, in the group IV animals the liver seemed particularly susceptible to iron accumulation.

As is also shown in Table I, the estimated rate of daily iron absorption was higher in both groups of rats fed mealie-meal when compared with their appropriate stock diet controls. Especially notable, perhaps, is the high iron absorption in the rats fed mealie-meal alone (group II) despite the very low iron content of this diet (Table I). Enriching mealie-meal with iron considerably enhanced the iron uptake even though the daily food and iron intake in this group (group IV) was half that of group III.

Especially noteworthy were the contributions to the total body iron from the various organs in the different groups of rats. Text-figure 1 displays in a striking way the very much greater susceptiblity to siderosis of the livers of rats fed mealie-meal and iron. The quantitative findings fully confirm the histologic data outlined above.

Discussion

Observations indicate that dietary iron overload leads to an increase in the total body iron whether animals are consuming a "good" or a "bad" diet. However, rats fed the iron-enriched mealie-meal diet (group IV) became siderotic much more rapidly and more severely than rats fed a similarly enriched "good" diet. The "mucosal block" for iron, while not complete, is nevertheless more efficient in well-nourished than in malnourished rats. Whether the far greater iron uptake in rats fed mealie-meal and iron is a function of the phosphate, calcium, amino acid, or other dietary components, operating in the lumen of the intestine, as suggested by others,[10,17] still remains to be determined with the aid of radioactive iron. It still seems possible, however, that alterations in the mucosal block may perhaps be a reflection of profound changes in the intermediary metabolism of iron (and possibly of other substances, too) in cells throughout the body, but perhaps particularly in the liver.

In the rats fed mealie-meal and iron whose organs ultimately became heavily siderotic, iron-containing pigment was detectable, in notable quantities, first within the hepatic epithelium and only much later in sinusoidal or portal phagocytes. Moreover, even when the liver was severely siderotic, and when the hepatic iron had increased 6 or 7 fold, very little was visible in the epithelium of other parenchymal organs.

In this regard, dietary siderosis in the rat resembles human siderosis, for Gillman and Gillman[1,2] described iron as being detectable along the biliary poles of the liver cells in the earliest phase of African siderosis. This seems to be the pattern in other types of experimental dietary siderosis (cat[7]) and differs from siderosis induced by injecting iron, where this metal accumulates first in the reticuloendothelial cells in both the rat and the cat, but not in the rabbit.[18] If siderosis is studied in the late stages only, it becomes impossible, on histologic grounds, to determine where iron accumulates first, since, in advanced siderosis, both epithelial and phagocytic cells become laden with this metal. It seems that in African siderosis, as in dietary siderosis in rats, iron appears first in hepatic epithelium and only in the later stages in hepatic phagocytes.

It is also our impression that, as in our rats, so too in Africans, the epithelial cells of other organs, e.g., pancreas, salivary glands, and kidneys, become laden with iron only late in the disease but before the phagocytes of these organs. However, the spleen, lymph nodes and bone marrow, which contain only reticuloendothelial elements and are devoid of epithelium, may and usually do become siderotic fairly early. It is also noteworthy that in rats on an iron-enriched stock diet, the hepatic epithelium is free of histochemically demonstrable iron even though the total iron content of the liver, as estimated chemically, is twice that of the controls.

Our figures thus reveal the liver to be liable to iron accumulation to a greater extent than other organs, and especially so when the animals are malnourished.

It is not yet known whether, in idiopathic hemochromatosis, the epithelium or the phagocytes are the first to accumulate iron, simply because most tissue from such cases has been obtained at necropsy and late in the disease. Hence, biopsy specimens of the liver and other tissues from young individuals inheriting the hemochromatotic metabolic disorder, as evinced by plasma iron abnormalities, would be of fundamental importance. Very early stages of African siderosis have already been studied.[2] Until material becomes available from subjects in the earliest stages of idiopathic hemochromatosis, comparisons of these two human diseases, on the basis of the distribution of iron between epithelium and phagocytes of parenchymal organs, cannot permit conclusions as to their pathogenesis.

The massive accumulation of iron in hepatic epithelium and its retention in the liver (even at a later stage in the experiments) certainly seem to indicate that some alteration has occurred in the movements of iron throughout the body in the malnourished rats. This

possibility is supported by the heavy colonic siderosis in rats fed iron-enriched mealie-meal diets. Since excretion of endogenous iron increases, if the total iron content of the body and hence the turnover is greater,[19] this heavy colonic siderosis may perhaps indicate increased excretion of iron by our rats. However, this point can be settled only with the aid of radioactive isotopes.

It has been shown that the rate of iron uptake from the intestine may be altered in at least the following ways: (a) by increasing the dietary iron content; (b) by changing the dietary phosphate, calcium, protein, or amino acid contents; (c) by modifying the vitamin C or E content of the diet; (d) by acute or prolonged simulated high altitude (decompression anoxia).

It has also been suggested that the increased iron uptake and the better utilization of absorbed iron, induced by vitamin C and especially by vitamin E, is attributable to improved cellular oxidation-reduction systems, especially in the bone marrow, but perhaps also in the intestinal mucosa. Beilig and Bayer[20] have demonstrated the direct uptake of ferrous iron by apoferritin to form ferritin in the presence of oxygen, while Mazur, Baiz and Shorr[21] have shown that iron is released from ferritin in tissue slices under anaerobic conditions in the presence of ascorbic acid, glutathione, cysteine or anaerobic liver tissue. Loewus and Fineberg[22] further revealed that under aerobic conditions, and in the presence of ascorbic acid or boiled liver extracts, ferric iron may be reduced, adsorbed onto apoferritin and re-oxidized by atmospheric oxygen to ferritin. Furthermore, Takeda and Hara[23] maintained that the primary lesion in ascorbic acid deficiency is the mobilization of ferrous iron causing an inactivation of the enzymes which require the ferrous ion as a co-factor; this alteration is accompanied by a decrease in the activities of other enzymes leading, *inter alia*, to a depression of synthetic reactions including enzyme formation, with consequent quantitative decline in mitochondrial enzymes as a whole.

In the light of all the above, it is not surprising that artificially induced anoxia (decompression) rapidly causes a greater uptake of a single dose of ingested iron[24] and is soon associated with a distinct increase in circulating siderophilin and a drop in plasma iron content.[25] The well-known increase in the size of the circulating erythron, accompanying acclimatization to high altitudes, is possibly the later outcome of these initial changes, perhaps facilitated by an increase in circulating "erythropoietin."[26]

Thus, anoxia and several nutritionally induced intracellular metabolic disorders can and apparently do promote profound and wide-

spread alterations in tissue metabolism, all of which affect the activity and synthesis of mitochondria and especially of their iron-containing enzymes. Perhaps similar alterations in intracellular metabolism throughout the body, induced by the inadequate maize-meal diet, are ultimately responsible for the considerable changes noted in the absorption, transport, distribution and even in the intermediary metabolism of dietary iron in our experiments. How far these metabolic changes may exert their effects on iron absorption and metabolism through the "erythropoietin" hormone described by Jacobson, Goldwasser, Fried and Plzak[27] seems worthy of further study.

We have shown that consumption of a maize diet markedly increases the uptake of iron from the intestine and alters its distribution in the body. Gillman and Gillman suggested from their cytologic studies[28] that the earliest detectable lesions in African siderosis seemed to be in and around the hepatic mitochondria. It is interesting to note that this early opinion has been confirmed by Richter's recent electron microscope study which also indicates that "hemosiderin is derived from altered mitochondria or 'siderosomes'."[29] The biochemical data quoted above and our present experimental findings would seem to support the Gillmans' contention that some nutritionally conditioned intracellular metabolic disorder (or disorders) seems to underlie the excessive absorption and utilization of dietary iron and its consequent deposition in the liver and other tissues in Africans with siderosis. Moreover, the cytologic evidence, coupled with our chemical findings and other data from the literature,[29,30] provide strong indications that many derangements in iron metabolism (including microcytic anemia) previously attributed solely to peculiarities in iron intake, may be shown to be due rather to widespread, initially intracellular metabolic disorders, resulting, in many instances, from one or another form of malnutrition.

Perhaps the accumulation of iron pigment within the liver cells, like the accumulation of fat, may be the end result of a variety of metabolic lesions acting through a single final common intracellular path. Whereas fatty change is known to be one morphologic expression of several distinct derangements in fat metabolism, so too, perhaps, siderosis may result from disturbances in the rate of synthesis and destruction of iron-containing enzymes, perhaps primarily in the liver. Recent reviews[29] of various aspects of iron metabolism indicate that, contrary to previous expectations, intracellular iron enzymes may be far more susceptible than the circulating erythron to peculiarities in dietary iron content. Of particular interest in this connection, is Beutler's observation[30,32] of a fall in cytochrome C content of the liver

and kidney of rats on an iron deficient diet before anemia developed. This further underlines the susceptibility of intracellular iron-containing enzymes, and the mitochondria with which they are associated, to alterations in metabolism resulting from various forms of malnutrition.[31]

All the present indications are that, as first suggested by Gillman and Gillman in 1944,[28] some lesion involving the iron-containing enzymes, located in or on the mitochondria, is to be anticipated as the underlying basis for increased uptake of dietary iron so clearly induced by a "bad" diet. Such mitochondrial lesions could perhaps be produced by anoxia or at least by intermittent cellular hypoxia. The effects on plasma iron and iron uptake of a single brief exposure to hypoxia may persist for several days.[24,25] Using this knowledge, we have now completed further experiments, the preliminary results of which demonstrate clearly that short, intermittent decompression (at 360 mm. of Hg for 1½ hours twice weekly) markedly increases the rate and amount of iron absorbed and stored in the liver of rats fed an iron-enriched maize diet, but has very little effect on rats fed the stock diet plus iron. In rats fed the mealie-meal and iron diet, but with the calcium and phosphorus content equal to the stock diet plus iron, intermittent decompression exerts effects on hepatic iron which are intermediary between the groups just mentioned.

These recent experiments demonstrate that intermittent hypoxia markedly augments the amount and speed of iron uptake by rats on an iron-rich but otherwise "bad" diet, but not by rats on a "good" diet even if heavily laden with iron. The intermediary metabolic effects of the "bad" diet and of intermittent hypoxia seem to act synergistically, promoting speedy and excessive iron absorption and deposition in tissues, initially especially in hepatic cells.

The results of our decompression experiments will be fully reported elsewhere. Yet we feel justified, at this stage, in suggesting that the basic alteration promoting excessive iron uptake in subjects consuming a mealie-meal diet, is not the high iron intake alone. The low calcium and especially phosphate content, coupled particularly with intracellular hypoxia or anoxia (perhaps intermittent, due to seasonally linked malnutrition in man—e.g., pellagra), seem to be of fundamental importance in iron metabolism.

Whatever the mechanism(s), however, one thing seems clear from the foregoing experiment. It is far easier to produce an increased iron uptake and demonstrable hepatic siderosis by dietary iron overload (coupled with intermittent hypoxia) in malnourished than in well-fed animals.

Summary and Conclusions

Histochemical and quantitative chemical data reveal that the uptake of iron from iron-enriched diets, is much greater in rats fed a "bad" (maize-meal) diet than those fed a "good" (stock) diet. This increased iron uptake by rats consuming iron-enriched mealie-meal is reflected in the far greater total carcass iron despite the smaller daily and total iron ingested. It is also characterized by lesser growth of these rats when compared with other groups.

Dietary siderosis in rats is characterized by an accumulation of histochemically demonstrable iron in liver cells before it appears in hepatic phagocytes or other parenchymal cells. Iron absorbed in excess seems to accumulate in the liver to a large extent before other non-reticuloendothelial organs become notably siderotic. In these respects the lesions in rats resemble those in the early stages of African dietary siderosis.

The colon becomes heavily laden with iron whereas the duodenum remains virtually free of it even in heavily siderotic rats.

The histochemically and chemically determined distribution of iron in these experiments, taken in the light of data from the literature, adds additional information in support of the possibility that the underlying lesion in nutritional siderosis pertains to the intracellular iron-containing enzymes located on the mitochondria. The role of intracellular hypoxia (or anoxia), perhaps induced by malnutrition in increasing the speed and degree of iron uptake and the onset of hepatocellular siderosis, is discussed in the light of the present results.

It is concluded that dietary siderosis in rats and, perhaps, in man, too, is not due to iron overload alone but may rather be attributable to some effects of a "bad" diet on intracellular metabolism.

REFERENCES

1. Gillman, J., and Gillman, T. Structure of the liver in pellagra. *Arch. Path.*, 1945, **40**, 239–263.
2. Gillman, J., and Gillman, T. Perspectives in Human Malnutrition. A Contribution to the Biology of Disease from a Clinical and Pathological Study of Chronic Malnutrition and Pellagra in the African. Grune & Stratton, New York, 1951, 584 pp.
3. Walker, A. R. P., and Arvidsson, U. B. Iron "overload" in the South African Bantu. *Tr. Roy. Soc. Trop. Med. & Hyg.*, 1953, **47**, 536–548.
4. Higginson, J. The liver in haemochromatosis. (Letter to the editor.) *Lancet*, 1957, **1**, 994–995.
5. Steinkamp, R.; Dubach, R., and Moore, C. V. Studies in iron transportation and metabolism. VIII. Absorption of radioiron from iron-enriched bread. *A. M. A. Arch. Int. Med.*, 1955, **95**, 181–193.

6. Kinney, T. D.; Hegsted, D. M., and Finch, C. A. The influence of diet on iron absorption. I. The pathology of iron excess. *J. Exper. Med.*, 1949, **90**, 137–146.

7. Taylor, J.; Stiven, D., and Reid, E. W. Haemochromatosis in a depancreatised cat. *J. Path. & Bact.*, 1931, **34**, 793–797.

8. Rather, L. J. Hemochromatosis and hemosiderosis. Does iron overload cause diffuse fibrosis of the liver? *Am. J. Med.*, 1956, **21**, 857–866.

9. Hegsted, D. M.; Finch, C. A., and Kinney, T. D. The influence of diet on iron absorption. II. The interrelation of iron and phosphorus. *J. Exper. Med.*, 1949, **90**, 147–156.

10. Factors affecting iron absorption. *Nutrition Rev.*, 1958, **16**, 113–114.

11. Dry, D. S. Improved methods for the demonstration of mitochondria glycogen, fat and iron in animal cells. *South African J. Sc.*, 1945, **41**, 298–301.

12. Gillman, J., and Gillman, T. Anoxia and the liver with special reference to shock and chronic malnutrition. *Am. J. Digest. Dis.*, 1949, **16**, 348–372.

13. Gillman, T. Nutrition, liver disease and some aspects of ageing in Africans. Ciba Foundation Colloquia on Ageing. 3. Methodology of the Study of Ageing. Little, Brown & Co., Boston, 1957, **3**, 104–114; 188–190.

14. Gillman, T.; Hathorn, M., and Lamont, N. Liver disease in Durban Africans. Histological findings in biopsies. *South African J. Med. Sc.*, 1959, (in press).

15. Gillman, T., and Ivy, A. C. A histological study of the participation of the intestinal epithelium, the reticulo endothelial system and the lymphatics in iron absorption and transport. Preliminary report. *Gastroenterology*, 1947, **9**, 162–169.

16. Granick, S. Ferritin. IX. Increase of the protein apoferritin in gastrointestinal mucosa as a direct response to iron feeding. The functions of ferritin in the regulation of iron absorption. *J. Biol. Chem.*, 1946, **164**, 737–746.

17. Chapman, D. G., and Campbell, J. A. Effect of calcium and phosphorus salts on the utilization of iron by anaemic rats. *Brit. J. Nutrition*, 1957, **11**, 127–133.

18. Goldberg, L.; Smith, J. P., and Martin, L. E. The effects of intensive and prolonged administration of iron parenterally in animals. *Brit. J. Exper. Path.*, 1957, **38**, 297–311.

19. Bothwell, T. H., and Finch, C. A. The intestine in iron metabolism. Its role in normal and abnormal states. *Am. J. Digest. Dis.*, 1957, **NS2**, 145–158.

20. Bielig, H. J., and Bayer, E. Synthetisches Ferritin, ein Eisen(III)-komplex des Apoferritins. *Naturwissenschaften*, 1955, **42**, 125–126. Cited by Loewus and Fineberg.[22]

21. Mazur, A.; Baez, S., and Shorr, E. The mechanism of iron release from ferritin as related to its biological properties. *J. Biol. Chem.*, 1955, **213**, 147–160.

22. Loewus, M. W., and Fineberg, R. A. The incorporation of iron by apoferritin. *Biochim. et. biophys. acta.*, 1957, **26**, 441–443.

23. Takeda, Y., and Hara, M. Significance of ferrous ion and ascorbic acid in the operation of the tricarboxylic acid cycle. *J. Biol. Chem.*, 1955, **214**, 657–670.

24. Smith, D. L.; Bierwagen, M. E., and Early, T. K. Effect of environmental oxygen tension on intestinal iron absorption. *Proc. Soc. Exper. Biol. & Med.*, 1957, **94**, 710–711.

25. Colehour, J. K.; Borsook, H., and Graybiel, A. Effect of hypoxia on serum iron and the unsaturated iron-binding capacity of serum in rats. (Abstract:) *Am. J. Physiol.*, 1957, 191, 113–114.

26. Goldwasser, E.; Jacobson, L. O.; Fried, W., and Plzak, L. F. Studies on erythropoiesis. V. The effect of cobalt on the production of erythropoietin. *Blood*, 1958, 13, 55–60.

27. Jacobson, L. O.; Goldwasser, E.; Fried, W., and Plzak, L. Cited by Goldwasser *et al.*[26]

28. Gillman, T., and Gillman, J. Mitochondrial origin of cytosiderin (iron pigment) in the liver of human pellagrins. (Letter to the editor.) *Nature, London*, 1944, 154, 148.

29. Iron stores and iron deficiency. (Editorial.) *Brit. M. J.*, 1958, 1, 1167–1168.

30. Cytochrome C levels in iron deficiency without anaemia. *Nutrition Rev.*, 1958, 16, 155–157.

31. Gillman, T. Cell enzymes and iron metabolism in anemias and siderosis. *Nutrition Rev.*, 1958, 16, 353–355.

32. Beutler, E. Iron enzymes in iron deficiency. I. Cytochrome C. *Am. J. M. Sc.*, 1957, 234, 517–527.

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LEGENDS FOR FIGURES

All sections have been stained to demonstrate iron.

FIG. 1. Liver from rat fed maize-meal plus iron for 50 days, showing moderate amount of iron primarily in periportal epithelial cells. × 30.

FIG. 2. Higher power view of liver in Figure 1, showing majority of iron as fine granules in somewhat vacuolated (non-fatty) periportal liver cells. There are minimal amounts in sinusoidal phagocytes. × 85.

FIG. 3. Liver of rat fed maize-meal and iron for 109 days, showing clumping of iron in hepatic cells, especially periportally, and now also in sinusoidal and portal phagocytes. × 85.

FIG. 4. Low power view, showing heavy siderosis of liver cells as well as sinusoidal phagocytes in a rat fed iron-enriched maize-meal diet for 156 days. Note that periportal liver cells are still most severely siderotic. × 30.

FIG. 5. Higher power view of liver in Figure 4 to show clumping of iron within liver cells and the extent and distribution of iron in phagocytes (compare with Figure 6). × 85.

FIG. 6. Section of liver typical of control rats fed iron-enriched stock diet for 155 days. Note absence of histochemically demonstrable iron in liver cells and only small granules in occasional sinusoidal and portal tract phagocytes. × 85.

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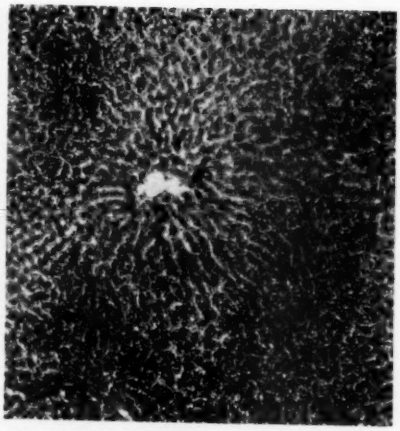

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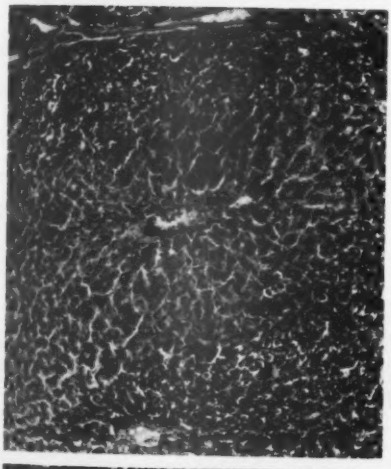

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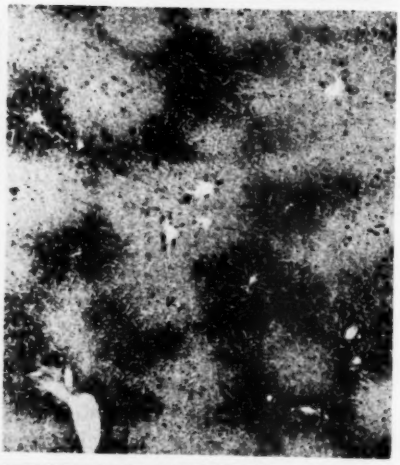

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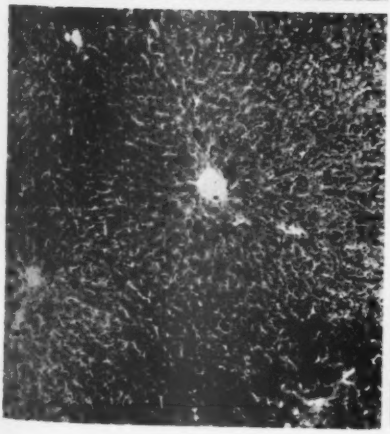

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FIG. 7. High power view of mid-lobular liver cells to show fine intracellular iron-containing granules near the biliary poles of the cells; also moderate amounts of iron in sinusoidal phagocytes. Rat fed maize-meal plus iron for 155 days. × 375.

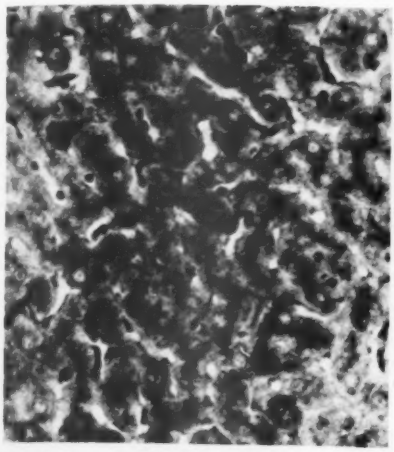
FIG. 8. Same section as Figure 7 but showing details of essentially biliary location of the fine iron-containing granules in the liver cells. This appearance is not easily distinguishable from that seen in the liver in the early stages of African siderosis. × 830.

FIG. 9. Diffuse distribution and clumping of iron within the more severely siderotic periportal liver cells in another rat fed maize-meal plus iron for 156 days. × 375.

FIG. 10. Vacuolation (non-fatty) of liver cells, resembling that described in anoxic livers, together with fine and coarse iron granules in liver cells of rat fed maize-meal plus iron for 50 days. × 830.

FIG. 11. Spleen of control rat, fed stock diet plus iron for 154 days, showing moderately severe siderosis of pulp and malpighian corpuscles. Compare with Figure 12. × 30.

FIG. 12. Spleen of rat fed maize-meal plus iron for 156 days. Both the pulp and the corpuscles seem to be somewhat more severely siderotic than in the control spleen (Fig. 11). Clear differences cannot be established with histochemical criteria although quantitative chemical data do show distinctly greater splenic iron in rats consuming mealie-meal plus iron. × 30.



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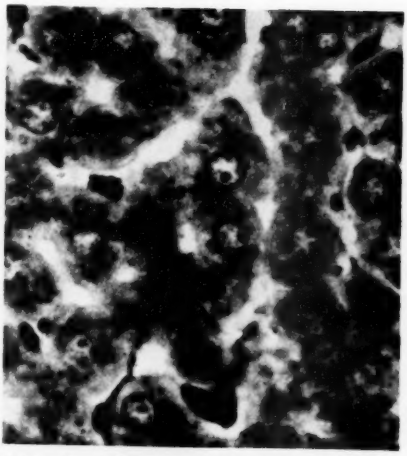

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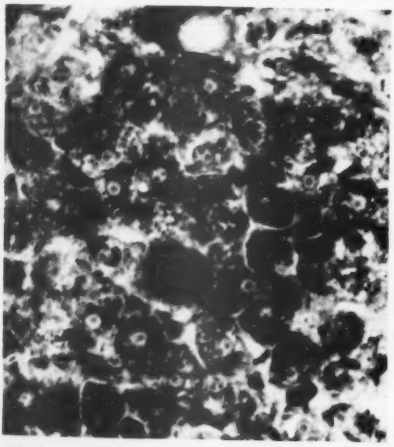

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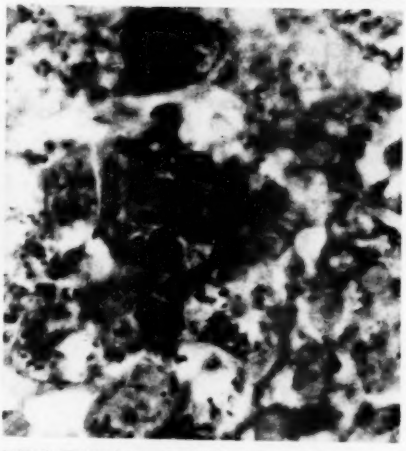

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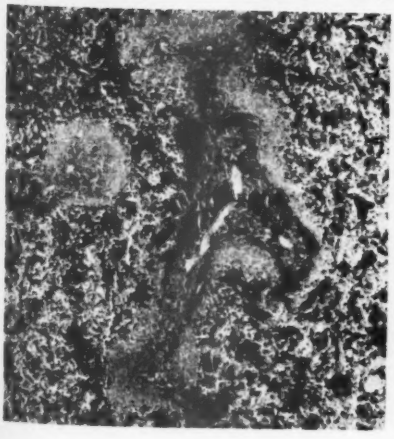

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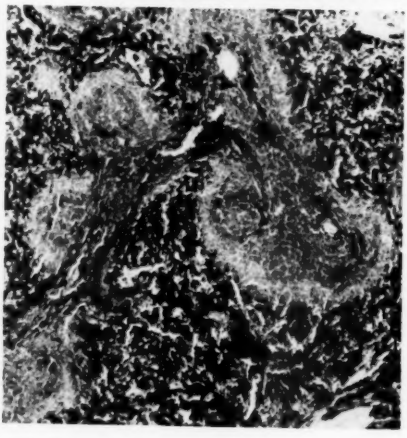

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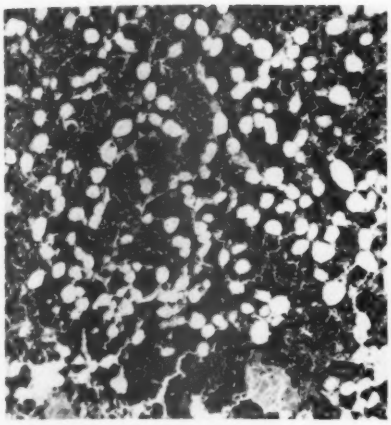
FIG. 13. Femoral bone marrow of rat fed stock diet plus iron for 155 days. Note numbers and sizes of iron-containing (dark black) cells. Compare with Figure 14. × 85.

FIG. 14. Femoral marrow of rat fed maize-meal plus iron for 155 days. Comparison, with a ruler, indicates greater numbers of larger iron-containing phagocytes in any field here than in the control rat marrow exemplified in Figure 13. × 85.

FIG. 15. Typical appearance of colon of rat fed stock-diet plus iron for 155 days, with minimal amounts of iron in either the epithelium or the phagocytes of the *tunica propria*. Compare with Figure 16. × 85.

FIG. 16. Moderate siderosis (fine granules) of colonic surface epithelium and massive siderosis of phagocytes, especially in the *tunica propria* but also in the submucosa, typical of rats fed maize-meal plus iron for 155 days. × 85.

FIG. 17. Typical appearance of duodenum in rats fed maize-meal plus iron for 156 days. Note virtual absence of iron from epithelium of mucosa and submucosal Brunner's glands and even from the phagocytes in the *tunica propria*. This is in striking contrast to the colon. × 85.



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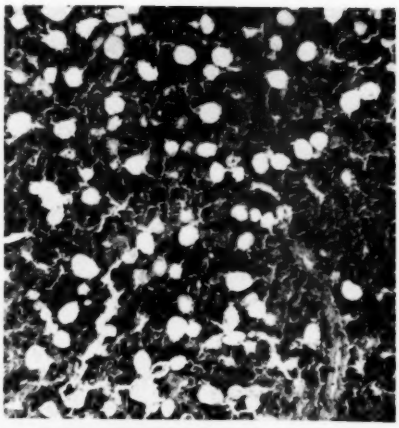

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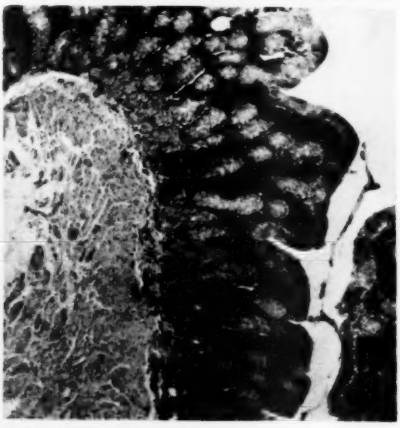

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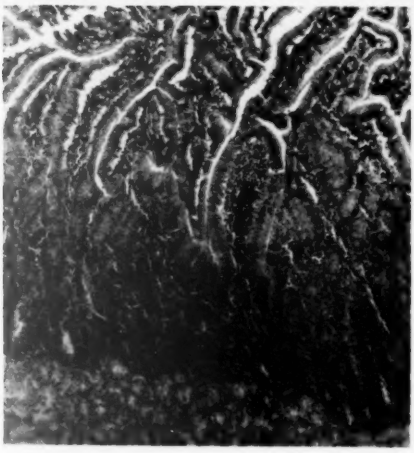

17

CELL DEATH

I. The Effect of Injury on the Proteins and Deoxyribonucleic Acid of Ehrlich Tumor Cells *

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The exact point at which death of a cell occurs is unknown. Although it is possible at certain stages to say of a cell that it is "alive" or "dead," the transition period between these two stages is as yet ill defined. It is not known which function or chemical reaction, when lost, is the first cause of irreversible damage to structural and metabolic integrity. Perhaps there exists a hierarchy of cellular phenomena, some of which are expendable, some replaceable, and others indispensable. These irreplaceable phenomena may correspond to the nucleic acids and proteins in the simplest forms of viruses. Only in certain structural combinations may they be said to constitute "life."

It is the purpose of this investigation to determine which of several cellular processes measured is most susceptible to injury. In the future it would be of interest to discover the point at which damage is irreparable and to determine what can be done to prevent, delay, or reverse the consequences of injury.

Many studies of biochemical and physiologic functions of cells require removal from their normal environment. The cells are not only placed in media which do not exactly duplicate their original surroundings, but are also removed from hormonal, nervous, and other homeostatic regulating influences of the intact organism. An acute insult to a cell may be as subtle as the omission of an essential constituent in the medium or, conversely, supplying one factor in toxic amounts. Changes in pressure, temperature, and pH are among the more common types of injury employed in altering a cell's environment. Recently, chemicals with more or less specific actions, such as metabolic analogues and inhibitors, have been used extensively.

The experiments in this laboratory have been designed to study the death of cells killed by 3 methods. In the first, death of control cells has been brought about by inability to reproduce exactly the environment under which the cells live in the organism. In addition, they lacked sufficient amino acids and substrates to survive at a normal metabolic rate for a prolonged period of time. The second and third

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methods of injury were selected to aggravate the above insults and deficiencies and to bring about a more rapid alteration in those structural components essential for specific metabolic functions. The second injurious agent used was x-irradiation. The number of water molecules is vast compared to all other molecules in the cell and its environment, and thus water has been studied as the molecule most likely to be ionized. The work of Weiss[1] in relation to this "activated water theory" has been supplemented by Barron's ideas[2] on the importance of thiol groups, as well as the studies of Uretz, Zirkle and Bloom[3,4] concerning the direct effects of protons on chromosomes. The third injurious agent, salyrgan ($C_{13}H_{16}O_5NNaHg$), is a mercurial which inhibits all SH enzymes, including those of the glycolytic and respiratory pathways. It thus deprives the cell of energy vitally needed to maintain its dynamic state.

Gray[5] described the striking similarity of the injury produced by radiation and radiomimetic drugs, sulfur and nitrogen mustard. Although he believed that the primary effects of the latter agents might be different, the end results, especially in the process of mitosis, appeared identical. Our own observations of the end effects of irradiation and of a chemical inhibitor, salyrgan, not generally classified as radiomimetic, have completely confirmed this impression.

The cell selected for our experiments was that of the Ehrlich ascites tumor. The revival of interest in this tumor by Klein[6] in Sweden and Goldie and Felix[7] in the United States has prompted further study by many investigators. The tumor has been used quite extensively as a test system in the investigation of chemotherapeutic agents in malignant neoplasms.[8] A few authors[9,10] have studied some of the effects of irradiation on these cells.

Experiments

Approximately 100 Swiss albino mice received intraperitoneal injections of Ehrlich ascites tumor cells (0.1 cc. of a 5×10^6 cells per cc. concentration). Six days after injection, the cells were withdrawn by peritoneal puncture, washed 3 times and finally suspended in a modified Krebs-Ringer solution to a final concentration of 5×10^6 cells per cc.; glucose was added to a concentration of 0.0014 M. The proper dilution factor was obtained by direct cell count using a standard hemacytometer. The count was also checked by light absorption of the suspension as measured in a Coleman Junior Spectrophotometer.

The final cell suspension (usually 400 to 500 cc.) was divided into 3 equal portions. One sample was kept as a control specimen. The second sample received a surface dose of 1×10^6 r. of soft x-irradiation, and a mercurial inhibitor was added to the third.

For irradiation of cells, AEG and OEG Picker roentgen units were used at 50 kv; 18 ma; TSD 8 cm.; no filter. The cells were placed in flat, siliconized Petri dishes, containing a siliconized glass-covered iron stirrer. A Labline electromagnetic stirring apparatus was placed under the Petri dish to assure a continuous, homogeneous suspension of cells throughout the 40 minutes of irradiation. Chemical dosimetry measurements of 2,3,5-triphenyltetrazolium chloride solutions handled in an identical manner indicated that the average dose received by the cells *in vitro* was well over 500,000 r. (Table I).

TABLE I

Effect of X-irradiation (1×10^6 r.) on Ehrlich Tumor Cells in Varying Volumes of Solution

Sample no.	Solution volume (cc.)	Solution depth (mm.)	Effect of x-rays by percentage of surface incident dose	Dose received (r.)
1	0	0	100	1,000,000
2	25	5	79.4	794,000
3	30	6	75.1	751,000
4	40	8	66.8	668,000
5	50	10	58	580,000
6	60	12	50.1	501,000

All the samples were kept at room temperature until the completion of the irradiation. At this point a buffered solution of salyrgan was introduced into the third sample to a final concentration of 0.001 M; and 3 Erlenmayer flasks, containing the control, the irradiated, and the salyrgan-treated cells, respectively, were placed in a shaking water bath maintained at 37.5° C. Aliquots were removed at 4 one-half hour periods, followed by 4 one-hour periods from each of the 3 sample flasks, for multiple physiologic, biochemical, and morphologic determinations. The experiments were continued for varying lengths of time ranging from 5 to 18 hours. The results of the following procedures will be reported in this paper. Other investigations carried out concurrently will be reported in subsequent papers.

Procedures

Three one-cc. samples of cell suspension were centrifuged and the protein in the extracellular supernatant precipitated with 2 drops of 30 per cent sulfosalicylic acid. The resultant turbidity was then read in a Fisher Nefluoro-Photometer, using a 10 mg. per cc. solution of beef albumin as a standard.

Cell suspension (0.55 cc.) was placed in 2 cc. of Bouin's solution,

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fixed for 24 hours, centrifuged, and the sediment, decanted evenly on a slide, was stained with hematoxylin and eosin.

Aliquots (0.5 cc.) of cells obtained from the 3 sample cultures at different time periods were fixed in Carnoy's solution for 24 hours and centrifuged. The sedimented cells were decanted evenly on a slide and dried. All slides in a given experiment were stained as a group simultaneously by Feulgen's fuchsin-sulfuric acid reaction. Individual cells of equal diameter were selected, and the amount of deoxyribonucleic acid (DNA) per nucleus was measured comparatively by means of a microspectrophotometer. The DNA values obtained from the cells taken in the first aliquot were given the value of 100 per cent, and all values obtained from later samples compared on a percentage basis. Twenty nuclei of equal size were measured in each of the 3 sample flasks at several time periods and the results subjected to analysis of variance.

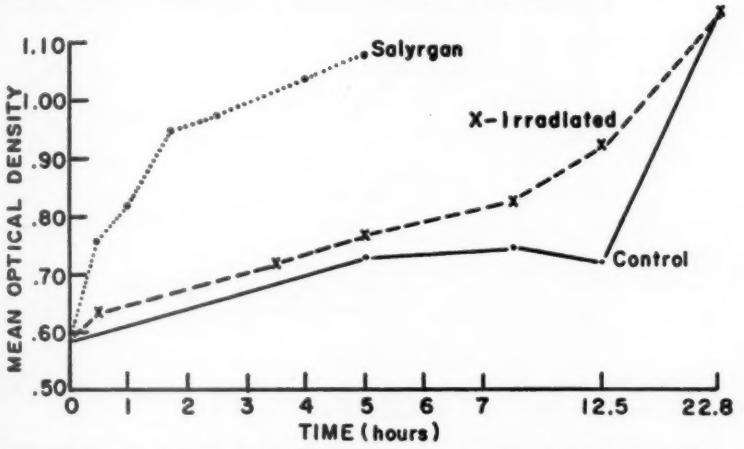
Nuclear sizes were determined by planimetry measurements of camera lucida drawings of cells stained with hematoxylin and eosin. All measurements were made on cells fixed and stained in an identical manner, and the figures given are units of the planimeter used.

Krebs-Ringer phosphate solution was used as an incubation medium. Ehrlich tumor cells placed in this medium will maintain the original number of cells for a period as long as 8 hours, while cells placed in a simple Sorenson's phosphate buffer start to disintegrate after one hour. Cells have been kept in Krebs-Ringer solution for as long as 18 hours at 37° C. without any damage as measured by the entrance of trypan blue into the cells. Locke's and Tyrode's solutions have also been satisfactory media.

Cell counts were determined in several different ways. A simple method of determining whether the original number of cells was present was by the use of trypan blue. In our experience, cells do not disintegrate without first taking up this dye. The absence of dye-stained cells indicated that the number of cells had not decreased from the original. Hemacytometer counts confirmed this impression. A quick and fairly accurate estimate of cell number may be gained by assaying the turbidity of a suspension of tumor cells by means of a Coleman Junior Spectrophotometer Nephelometer. However, the presence of dead or partially dead cells in the suspension contributes protein to the turbidity. These factors make this method inaccurate and highly misleading for experiments of long duration. In addition, changes in cell volume alter the amount of light transmitted through individual cells. Similarly, the amount of intracellular protein in the

cells of a given volume decreases with time. Thus dry weight may be correlated with cell numbers only in the initial sample.

The x-ray dose selected for these experiments was the smallest dose which would give any diminution of the dehydrogenase activity of the cell (as measured in the Thunberg tube) immediately following irradiation. This happened to be 1×10^6 r. air surface dose, as measured with a Victoreen chamber. The average dose received by the cells varied with the depth of the solution. The reduction of triphenyltetrazolium salt solutions of varying depths was used as an index of the penetration of the soft x-rays. Table I shows the average dose received by the cells in suspensions varying from 1 to 60 cc. Most of the experiments were carried out with 25 to 60 cc. of cell suspensions, indicating an average cell dose to be 501,000 to 794,000 r.



Text-figure 1. Effect of x-irradiation (1×10^6 r.) and salyrgan (0.001 M) on the release of protein from the cell into extracellular fluid. Each point represents the average of 3 determinations in a typical experiment.

It was never possible to produce transplanted tumors in mice given injections of cells receiving this amount of irradiation. Although in these experiments immediate irreversible mitotic depression was induced in Ehrlich cells given 1×10^6 r., it is logical to assume from the publications of others that this could have been accomplished with much smaller doses. The highest dose previously reported as necessary for mitotic inhibition in Ehrlich tumor cells was 10,000 r., a dose still far below that necessary to produce measurable effects in other metabolic activities.

The results of the spectrophotometric studies of the extracellular protein released from the cells are shown in Text-figure 1. The greatest

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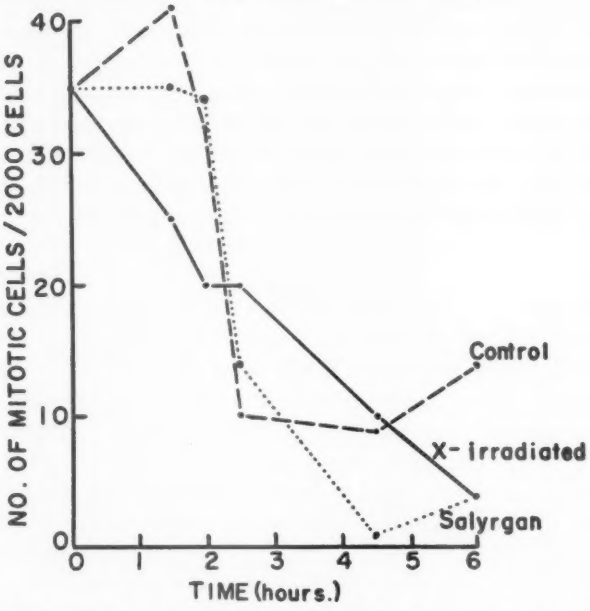
loss of protein was seen in the salyrgan-treated cells (Figs. 1 to 9). Cells stained with hematoxylin and eosin showed a definite loss of protein from both the nucleus and the cytoplasm. Although protein was lost from all 3 samples, that lost from the controls was the smallest when compared with the salyrgan and irradiated samples.



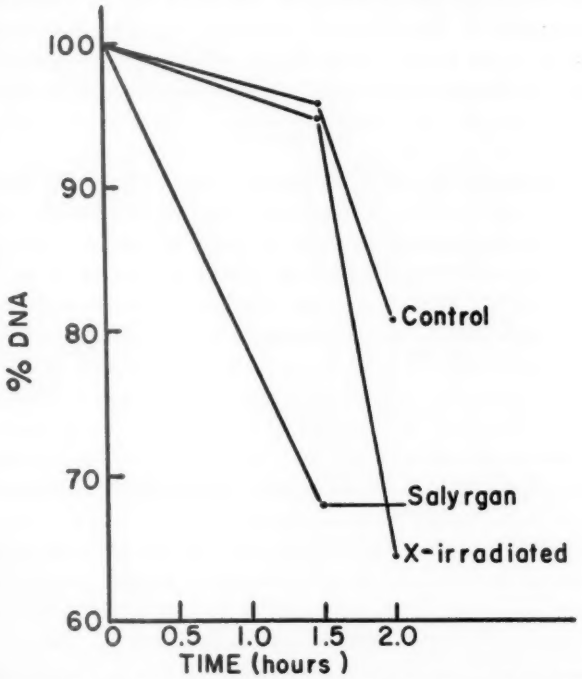
Text-figure 2. Effect of x-irradiation (1×10^6 r.) and salyrgan (0.001 M) on the mitotic rate of Ehrlich tumor cells in a typical experiment.

The mitotic indices of Ehrlich cells ranged from 1.3 to 2.1 per cent with an average value of 1.8 per cent. These figures compare very closely with those given by Lettré[11] (1.5, 1.0, and 1.1 per cent) and by Klein[12] (2.8 per cent) on the same tumor. Most workers recognize that the mitotic index is dependent upon the age and condition of the tumor cells when used. Cells removed from mice carrying the Ehrlich tumor for over 7 days had a greatly reduced mitotic index. It was interesting that all 3 forms of insult induced an immediate drop of over 50 per cent in the mitotic index, showing that the division mechanism is one of the most sensitive to injury (Text-fig. 2).

Aliquots of cells obtained from the 3 samples were removed, fixed, stained, and in every manner treated identically in preparation for examination under the microspectrophotometer. This method has been used to good advantage to show comparative values of DNA but is not

reliable to show absolute values. The readings obtained by measuring the absorption of the initial control samples were taken as 100 per cent. Text-figure 3 shows the immediate loss of nucleic acid from the cell nuclei of all 3 samples.

Usually it is desirable to use the microspectrophotometric method



Text-figure 3. Effect of x-irradiation (1×10^6 r.) and salyrgan (0.001 M) on DNA content of Ehrlich tumor cells in a typical experiment. Each point represents the average of determinations on 20 single nuclei. The drop in DNA in all 3 groups (control, x-irradiated, and salyrgan) is significant at the 1 per cent level by analysis of variance.

on a very homogeneous group of nuclei. Although from the photographs it would appear that Ehrlich tumor cell nuclei were of uniform size, it was apparent on careful study with the planimeter (on spherical nuclei only) that there was considerable variation. This factor becomes most important when a study is made of a group of cells undergoing related changes in water and electrolyte content which may alter the size of the nucleus markedly. A small, dark, shrunken nucleus may appear to have many times the DNA of a large, swollen nucleus with light, thinly scattered areas of Feulgen stained substance, although the total amount in both is the same. Thus, when samples are taken over

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a long period of time, the average nuclear size of the cell population must be taken into account. It is not enough to take only the same size cell on each successive sample. Readings on a control group of nuclei (25 μ in diameter) may not be the same as readings taken on a control group (25 μ in diameter) at a later time period. The latter may represent swollen nuclei (originally 20 μ in diameter) or shrunken nuclei (originally 30 μ in diameter). If a single size is used as a reference, false increases or decreases in DNA content will be recorded as a result of change in size and not as a result of change in nucleic acids.

Discussion

There was an obvious disruption of the protein and nucleic acid structure of a cell when it was subjected to a severe insult. One of the most interesting observations was the large amount of gross insult in addition to the obvious deleterious change in environment that was necessary to alter cell structure. It is well accepted that large parts of the kidney and liver in intact mammals may not be functioning at all times. It is less well accepted, but perhaps just as true, that large segments of the cell may be destroyed without irreversible damage. This may be due to a repetitive pattern of a cell or its innate reserves. Investigations in our laboratory with tissue culture cells have shown large alterations in the amount of protein per cell in a suspension of a pure strain at different stages in its growth.

Bensley,[13-15] using salt extractions, described a 70 per cent loss of a protein called plasmosin from cells without a change in their structure. Mirsky and Pollister[16] later repeated these experiments and identified the plasmosin substances with deoxyribonucleic acid. In our laboratory both points of view were entertained in the investigation of the protein released from the cell. Microspectrophotometric measurements did show a loss of DNA from the nucleus, and the nucleus appeared to become lighter in successive photographs of hematoxylin and eosin stained sections. However, other photographs of cells which were fixed, stained and photographed in an identical manner showed a concurrent lightening of the cytoplasm (Figs. 1 to 9).

The properties of a cell make its proteins different from those in a pure solution. The presence of resistant linkages, protected groups, and particularly the ability of the cell to resynthesize protein and repair damage, make it logical to assume that the cell would be very resistant to damage. However, a small change in the reactive grouping of a particular molecule, while not markedly altering the structure, may destroy its biologic effectiveness and thus eventually result in very severe injury.

Our experiments indicated that cells incubated in Krebs-Ringer medium exhibited an immediate appearance of and a continued increase in protein released into the suspending medium. The quantity of the protein was found to be distinctly related to the degree of injury suffered by the cell. The observations showed the immediate release of 30 per cent more protein into the medium from salyrgan-treated cells than from control cells. Following disruption of the cell membrane, approximately one hour after the administration of salyrgan, there was a gradual dispersion of the cytoplasmic protein into the surrounding medium. The irradiated and control cells also steadily lost protein in small quantities (20 per cent of the original amount present) over a period of 8 hours. There was no protein in the suspending medium originally, cell counts remained normal, and vital stains failed to show disintegrated products of dead cells. It must be concluded, therefore, that in both the control and irradiated cells there was a progressive breakdown and extrusion of cell proteins through the cell membrane in some form, a phenomenon beginning at the inception of incubation. The cause of this is unknown.

Summary

Ehrlich ascites tumor cells, when injured, immediately lose protein from the cytoplasm and protein and deoxyribonucleic acid from the nucleus. This occurs before any other alterations are noted in the cell. Associated with the loss of these substances there is an immediate drop in the mitotic index of the cell.

REFERENCES

1. Weiss, J. Possible biological significance of the action of ionizing radiations on nucleic acids. (Letter to the editor.) *Nature, London,* 1952, **169,** 460–461.
2. Barron, E. S. G. The role of free radicals and oxygen in reactions produced by ionizing radiations. *Radiation Res.,* 1954, **1,** 109–124.
3. Uretz, R. B.; Bloom, W., and Zirkle, R. E. Irradiation of parts of individual cells. II. Effects of an ultraviolet microbeam focused on parts of chromosomes. *Science,* 1954, **120,** 197–199.
4. Zirkle, R. E., and Bloom, W. Irradiation of parts of individual cells. *Science,* 1953, **117,** 487–493.
5. Gray, L. H. Some characteristics of biological damage induced by ionizing radiations. *Radiation Res.,* 1954, **1,** 189–213.
6. Klein, G. Use of the Ehrlich ascites tumor of mice for quantitative studies on the growth and biochemistry of neoplastic cells. *Cancer,* 1950, **3,** 1052–1061.
7. Goldie, H., and Felix, M. D. Growth characteristics of free tumor cells transferred serially in the peritoneal fluid of the mouse. *Cancer Res.,* 1951, **11,** 73–80.
8. Sugiura, K. Effect of various compounds on the Ehrlich ascites carcinoma. *Cancer Res.,* 1953, **13,** 431–441.

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9. Klein, G., and Forssberg, A. Studies on the effect of x-rays on the biochemistry and cellular composition of ascites tumors. I. Effect on growth rate, cell volume, nucleic acid and nitrogen content in the Ehrlich ascites tumor. *Exper. Cell Res.*, 1954, **6**, 211–220.

10. Lasnitzki, I. The effect of radiation on ascites tumor cells. *Brit. J. Radiol.*, 1954, **27**, 228–235.

11. Lettré, H. Zur Teilungsgeschwindigkeit der Zellen des Mäuse-Ascites-Tumors. *Naturwissenschaften*, 1943, **31**, 467–468.

12. Klein, G. Comparative studies on mouse tumors with respect to their capacity for growth as "ascites tumors" and their average nucleic acid content per cell. *Exper. Cell Res.*, 1951, **2**, 518–573.

13. Bensley, R. R. Plasmosin. The gel- and fiber-forming constituent of the protoplasm of the hepatic cell. *Anat. Rec.*, 1938, **72**, 351–369.

14. Bensley, R. R. Chemical structure of cytoplasm. *Science*, 1942, **96**, 389–393.

15. Bensley, R. R. Chemical structure of cytoplasm. *Biol. Symposia*, 1943, **10**, 323–334.

16. Mirsky, A. E., and Pollister, A. W. Fibrous nucleoproteins of chromatin. *Biol. Symposia*, 1943, **10**, 247–260.

LEGENDS FOR FIGURES

All preparations were stained with hematoxylin and eosin and photographed at a magnification of × 970.

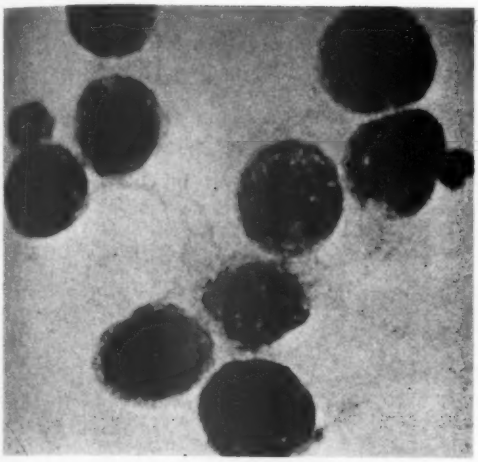
FIG. 1. Control cells immediately after incubation. They appear large, spherical, regular in outline and have a high nuclear cytoplasmic ratio.

FIG. 2. Control cells after 6 hours of incubation. The stain density of the nuclear and cytoplasmic protein appears similar to the initial control sample.

FIG. 3. Tumor cells immediately after irradiation. The cells appear very similar to the control cells seen in Figure 1.

FIG. 4. Two hours after incubation. The cytoplasmic stain density has lightened, indicating loss of protein. One cell has clumped chromosomes in metaphase.

FIG. 5. Six hours after incubation. The stain density is considerably lighter, showing loss of protein from both the nucleus and cytoplasm. The cell outlines are irregular and the cells have begun to swell, losing potassium and taking in sodium and water.



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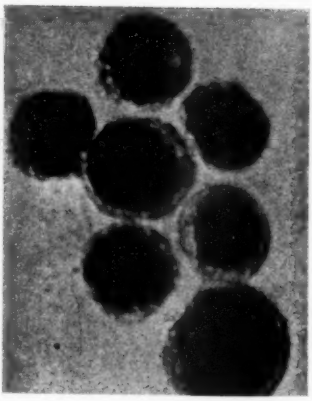

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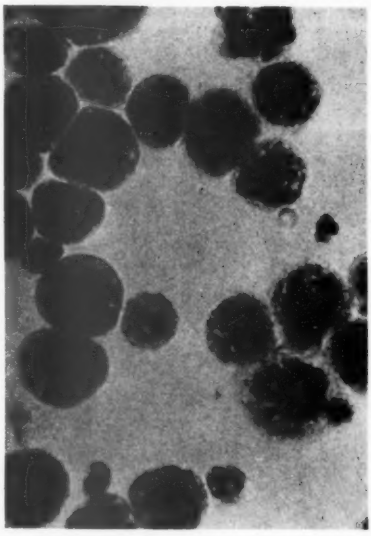

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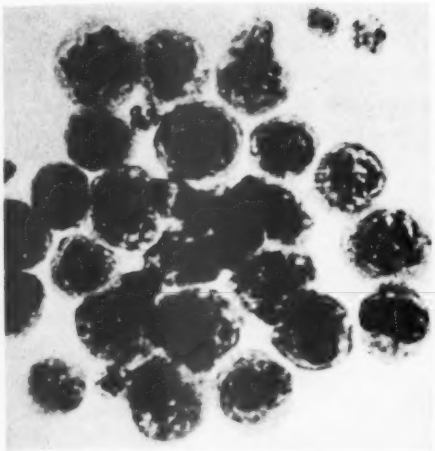
FIG. 6. Ehrlich tumor cells immediately after the addition of salyrgan. They appear similar to the controls seen in Figure 1.

FIG. 7. Thirty minutes after the addition of salyrgan. The cells are large and swollen. Their light staining indicates loss of protein from the cytoplasm and nucleus. In addition they have poor enzymatic activity and an electrolyte and water imbalance has begun.

FIG. 8. One hour after incubation. The cells have ruptured, releasing water, sodium, and protein to the outside environment. The cytoplasmic protein is gradually being dissolved into the surrounding medium. Many of the nuclei are compressed and shrunken, giving the false impression of increased amounts of nuclear substance.

FIG. 9. Six hours after incubation. The cells have dissolved completely, leaving a few remnants of their former structure. Conglomerate masses of eosinophilic denatured protein appear in clumps.



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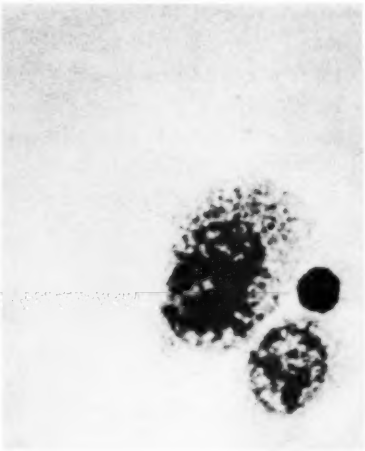

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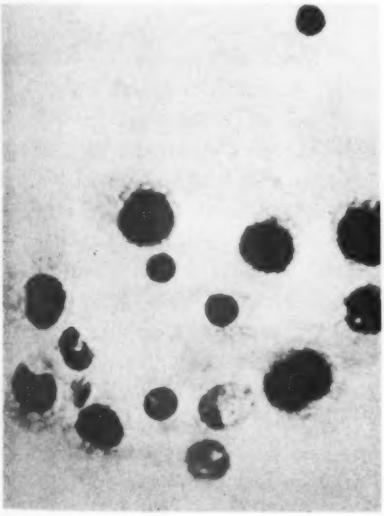

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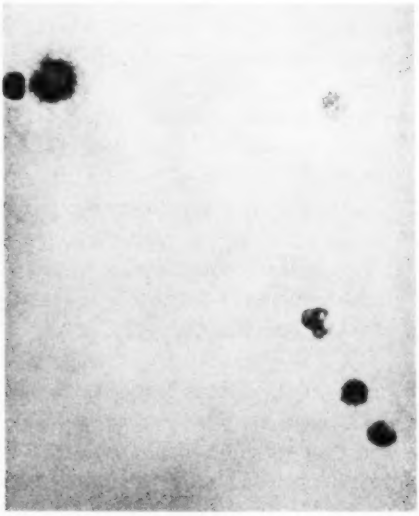

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TESTICULAR LESIONS FOLLOWING INGESTION OF DL-ETHIONINE STUDIED BY A QUANTITATIVE CYTOLOGIC METHOD*

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Severe testicular damage, which eventually ends in complete destruction of the germinal cells, has been observed in rats given ethionine orally or by injection.[1-3] The alterations appear to be similar to those which follow experimental deficiencies of a number of essential amino acids.[4-10] However, comparison of the earlier phases of the testicular lesions is difficult in view of the vague approximations used to characterize the cellular alterations by various groups of workers.

During investigations of the lesions produced by ethionine, a need was recognized for quantitative cytologic criteria to determine their severity. The method of Leblond and Clermont[11] lent itself readily to this purpose. In the study of testes of white rats fed DL-ethionine, counts of the various cell "association patterns" occurring during the maturation of spermatozoa were made according to this method.

MATERIAL AND METHODS

Male albino rats, random-bred at the Hebrew University, were used. These weighed about 120 gm. initially and were maintained on Purina Laboratory Chow (Ralston Purina Co., St. Louis, Missouri). The animals were divided into two groups; the first, comprising 5 rats, received tap water *ad libitum* with their food; the second group, also consisting of 5 rats, received DL-ethionine (Nutritional Biochemicals Corp., Cleveland, Ohio) as a 0.5 per cent solution in drinking water. The latter group ingested approximately 70 to 100 mg. of ethionine daily. Each animal was kept in a separate metabolic cage.

On the twelfth day of the experiment the animals were weighed, a left orchidectomy was performed under ether anesthesia, and the excised testis was examined histologically.

On the 24th day the rats were weighed and sacrificed by exsanguination. The remaining testis and slices of liver and pancreas were fixed in Zenker's acetic acid and 10 per cent formaldehyde solution. Paraffin sections were stained with hematoxylin and eosin (H and E), the periodic acid-Schiff (PAS) method counterstained with Ehrlich's hematoxylin, and methyl green pyronin.

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From the slices of each testis the average diameter of 20 cross sections of tubules, chosen at random, was calculated. In the same tubules the different stages of maturation were identified as outlined below.

It was established that unilateral orchidectomy had no effect on the weight or microscopic appearance of the remaining testis.

The Histologic Classification of Leblond and Clermont

Leblond and Clermont[11] demonstrated that spermiogenesis in the rat may be divided into 4 main phases. Each of these may be further subdivided into stages, of which there are 19 in all, identified by Arabic numerals.

The first or "Golgi phase" (stages 1 to 3) is characterized by the presence of an idiosome in the Golgi zone of the spermatid. This contains proacrosomic granules which condense into the acrosomic granule. In the second or "cap phase" (stages 4 to 7) the "head cap" develops from the acrosomic granule and covers one pole of the nucleus. In the third or "acrosome phase" (stages 7 to 14) the acrosome characteristic of the rat spermatozoa develops from the acrosomic granule. This is adjacent to the head cap and becomes oriented toward the basement membrane of the tubules. In the last or "maturation phase" (stages 15 to 19) the spermatids undergo development until released into the tubule lumen as free spermatozoa. The "maturation phase" spermatids are also referred to as "immature spermatozoa."

As the first 14 stages of spermiogenesis are well defined and easy to identify, they may be used to characterize the series of cell associations that make up one maturation "cycle" in the seminiferous epithelium. Each characteristic "cell association" is also designated a "stage" but is identified by Roman numerals I to XIV. In the cell association stages I to VIII, maturation phase spermatids (stages 15 to 19) were found constantly, and were accompanied by younger spermatids of stages 1 to 8. The existence of free spermatozoa and residual bodies, identified with the aid of the methyl green pyronin stain, was taken to indicate complete maturation of the germ cells. As suggested by Leblond and Clermont, the periodic acid-Schiff method was found useful in differentiating the 4 stages of the cap phase, clearly staining the head caps growing out of the acrosomic granules.

Results

Observations at 12 Days

All the animals receiving ethionine lost weight during the first 12 days on ethionine; the control animals gained 23 per cent in weight (Table I). The testes of the rats in the ethionine-treated group were

smaller than those of the controls, and the diameter of the seminiferous tubules was reduced (180 μ as compared to 200 μ; Table I). There was also deficient spermatogenesis, and it was impossible to identify all the stages described by Leblond and Clermont[11] because of the pathologic alterations. Therefore, a modification of the classification was found necessary.

After 12 days on ethionine no spermatids of stages 9 to 19 were found, but the tubules contained 3 easily identified cell association patterns. The first pattern was related to the Golgi phase spermatids (Fig. 1) and the second to the cap phase spermatids (Fig. 2). In the third pattern, neither the acrosome phase nor other spermatids were identified. Nevertheless, this pattern was easily recognized as related to the "cell associations" characterized by acrosome phase spermatids (stages 9 to 14) because of the presence of several layers of spermatocytes of different generations, ordinarily manifest in the "cell associations" IX to XIV.

The Golgi phase pattern (Fig. 1) was found in an average of 1 in 20 cross sections of the tubules (Table I). Very young spermatids, in which acrosomic granules could be barely distinguished, dominated the pattern (Fig. 1). Tubules of this type contained many Sertoli cells of the parallel type.[11] Neither giant cells, immature cells nor free spermatozoa were found in significant numbers.

Germ cell associations related to the cap phase spermatids were observed in 9 of 20 cross sections in the ethionine-treated group (Fig. 2; Table I). The caps appeared very thick and pleomorphic when stained by the periodic acid-Schiff method. The nuclei were also very irregular in size (Fig. 3) and some caps encircled the nuclei. Very few Sertoli cells of the parallel type were found in this pattern. Several layers of cap phase spermatids usually bordered the lumens, but very few germ cells of other types were encountered. (Fig. 2.).

In approximately 3 of every 20 cross-sectioned tubules, there were giant cells, formed by clusters of nuclei of cap phase spermatids (Figs. 4 and 5). The nuclei of the giant cells were either strung out around the periphery of the cells or scattered throughout the cytoplasm. No other type of giant cell was evident. Large vacuoles between the cells appeared to represent sites evacuated by degenerated giant cells (Fig. 5). In certain tubules there appeared to be a relationship between cap phase spermatids and many giant cells composed of cap nuclei (Fig. 4).

According to Leblond and Clermont,[11] stage VIII is characterized by 2 layers of spermatids. One of these represents maturation stage 8 (acrosome phase), in which the head caps and acrosomic granules be-

TABLE I

Effect of Ethionine
Testicular Injury and Weight Changes After 12 and 24 Days

	After 12 days		After 24 days	
	Group 1 P.L.C. and 0.5% ethionine	Group 2 P.L.C. and water	Group 1 P.L.C. and 0.5% ethionine	Group 2 P.L.C. and water
Number of animals	5	5	4*	5
Content of tubules (average of 20 cross sections)				
Golgi phase				
Stage I	1			
II	1			
III	1			1
Cap phase				
Stage IV	9	2		1
V	9	5		5
VI	9	2		1
VII	9	4		2
Acrosome phase				
Stage VIII	9†	3		4
IX	7	1		1
X	7			1
XI	7			1
XII	7	1		1
XIII	7	2		3
XIV	7			
Undifferentiated stage	3			
Average diameter, tubules (μ)	180	200	96	280
Residual bodies	3/20	10/20		8/20
Spermatozoa	very few	11/20		15/20
Average weight change (%)	−14.5	+23	−23	+37

P.L.C.: Purina Laboratory Chow.

* Anesthetic death of one animal during left orchidectomy on 12th day of experiment.

† Stage VIII designated as cap phase pattern because of similarity to cap phase spermatids.

come oriented towards the basement membrane of the tubules. The other represents stage 19 (immature) spermatozoa which line the lumen of the tubule and are released as free spermatozoa at the end of this stage. However, in the ethionine-treated group (Table I) this cell stage could no longer be identified. The immature spermatozoa (stage 19) were absent, and orientation of the head caps and acrosomic granules toward the basement membrane was not clearly evident. Moreover, the nuclei appeared round and were similar to those of the cap phase spermatids. It was, therefore, decided to assign the designation "cell association" stage VIII to the cap phase pattern (Table I).

The third pattern (Fig. 6) consisted predominantly of two generations of spermatocytes. An average of 7 tubules in 20 had a thin basal membrane with few Sertoli cells of the parallel type. In addition, there were several central layers of pachytene or diplotene spermatocytes and a peripheral layer of leptotene or zygotene spermatocytes. Maturation phase spermatids were practically absent, and no giant cells or free spermatozoa were visible (Fig. 6).

Maturation of germ cells is determined by the identification of residual bodies and of free spermatozoa. Only rarely were these elements found in the ethionine-treated rats. At 12 days the Leydig cells and the intertubular tissue showed no significant alterations.

In a certain number of tubules the "association stages" could no longer be differentiated because of the advanced state of the lesions (Table I).

Observations at 24 Days

All the animals receiving ethionine lost as much as 23 per cent of the original weight. Control rats gained up to 37 per cent of the original weight (Table I). It was noted that the rats receiving ethionine secreted much less urine than normal.

The testes of the ethionine-treated rats had become even smaller than those extirpated at 12 days. The average diameter of the seminiferous tubules was now about 96 μ (Fig. 7); the controls measured 280 μ (Table I; Fig. 8).

Cross section of the seminiferous tubule in the treated rats (Fig. 7) revealed many Sertoli cells of the parallel type near the basal membrane. There were no germ cells, giant cells, spermatozoa or residual bodies. In place of these elements were undifferentiated fibrils staining a pale bluish hue with hematoxylin and eosin. There was marked hyperplasia of the Leydig cells (Fig. 7).

In the controls normal spermatogenesis continued, and there were neither atrophy nor hyperplasia of the Leydig cells (Fig. 8).

At the conclusion of the experiment, the pancreas showed the anticipated severe lesions attributable to treatment with ethionine.[12,13] In the liver there were discrete lymphoid infiltrations in the portal spaces and a few fibroblasts and fibrocytes (Fig. 9). However, at this stage, the liver cells and the lobular pattern were still well preserved.

Discussion

Within the scope of this investigation, a quantitative evaluation of the extent of testicular damage induced by ethionine was made by a determination of "associations" of various histologic stages in maturing seminiferous epithelium. Since, however, the administration of ethionine for 12 days caused the elimination of the more mature

spermatids, the original classification proposed by Leblond and Clermont[11] had to be modified accordingly.

In the rats receiving ethionine for 12 days, no progress of maturation was found beyond cap phase spermatids (stage 8), whereas the more immature elements in the seminiferous epithelium appeared intact. There were also giant cells present as indicated by earlier investigators[1-3]; these authors had maintained that some of the giant cell nuclei resembled the nuclei of spermatids, and others, those of spermatocytes. However, the cytologic criteria utilized in the present study permitted the identification of all giant cell nuclei as those of "cap phase spermatids."

The observations suggest that after 12 days, ethionine caused the destruction of all acrosome phase spermatids and all spermatozoa, mature and immature, and arrested maturation between cell "association stages" VIII and IX. After 24 days, the destruction was much more extensive, so that only Sertoli cells remained in the tubules.

The lesions described appeared in all seminiferous tubules in a uniform manner. This observation contrasts with the experience of others[1] who, with the oral administration of ethionine, found the lesions to be less extensive, patchy, and less severe at corresponding experimental periods. Thus Kaufman, Klavins, and Kinney[2] observed complete destruction of the tubules only after 63 days. After 28 days, roughly corresponding to the length of our experiments, they found spermatogonia and occasional spermatids and spermatocytes in about half of their animals. In the remaining rats, spermatogenesis was irregularly affected. In occasional tubules complete maturation was still manifest. At 10 days, these authors still found spermatozoa, though in a reduced number, and there was only mild degeneration of spermatids.

It is presumed that the more severe lesions and weight loss observed in the rats in our experiments were due to the fact that ethionine was ingested in drinking water. This route may have permitted a greater intake of ethionine than was the case in the rats studied by Kaufman and co-workers[2] to which ethionine was given mixed in food. An additional factor may have been their use of older, heavier rats which may have had greater resistance than those in our experiments.

It has been proposed that the testicular damage in this circumstance may reflect ethionine-methionine antagonism.[2,14,15] Considerable degeneration of seminiferous tubules has been observed in rats maintained on a diet deficient in methionine for periods longer than 40 days.[16] However, the characteristic testicular lesions which follow the administration of ethionine do not appear to be specific for methionine

deficiency. They have also been reported in rats with induced deficiencies of other essential amino acids. [4–10,17]

Multinucleated giant cells in degenerating seminiferous tubules have been described repeatedly in rats following the experimental induction of acute and chronic inanition[18] and trytophan deficiency.[17] It has not been determined with certainty whether the multinucleated cells result from the fusion of spermatocytes or spermatids[1,17,18] or from defective division of cytoplasm following nuclear mitosis.[2,19] The consistent appearance of perfect cap phase spermatids, though permitting determination of the level at which the giant cells are formed, does not reveal the mode of their development.

Another feature in our specimens has been the regularity with which hyperplasia of Leydig cells has been noted at the end of the experiments. In previous studies Leydig cells have been reported to be unchanged during ethionine administration.[1,2] Moreover, although Maun, Cahill and Davis[4–6] found no changes in the Leydig cells in animals fed diets deficient in phenylalanine, leucine or histidine for periods of 28 days, others[7–10] observed atrophy of these cells in animals on similar diets for periods up to 42 days. Warren and Olshausen emphasized the varied results obtained in relation to interstitial cell hyperplasia in earlier investigations.[20] They suggested that hyperplasia of the Leydig cells might be more apparent than real, an effect induced by tubular atrophy and interstitial edema which often occurred in their experiments. On the other hand, in our material there appeared to be a true hyperplasia with numerical increase of Leydig cells.

The pathogenesis of the testicular lesions in this type of experiment has not been clearly established. Using ethionine labeled with radioactive sulfur (S^{35}), Fitzgerald, Hellman, Weinstein and Schimmel[21] demonstrated incorporation of the ethionine into protein formed in the testis, thus implying a direct toxic effect of the substance. This might conceivably be related to an endocrine imbalance since it is possible that, following destruction of germinal epithelium, the hypophysis might secrete less follicle-stimulating hormone (FSH). Hyperplasia of the Leydig cells could result from a compensatory hypersecretion of luteinizing hormone (LH).

Another explanation of the lesions proposed by Howard, Sniffen, Simmons and Albright[22] stems from studies of patients suffering from various testicular deficiencies. They and others[23–26] have suggested that the liver, damaged by ethionine, may be unable to destroy free circulating estrogens; there would follow decreased secretion of FSH and degeneration of the germinal epithelium. The X-hormone, secreted by the Sertoli cells and analogous in the male to estrogen,

may also cause inhibition of FSH secretion. The hypophysis, stimulated by these factors to increase its production of LH, may thus induce hyperplasia of the Leydig cells.

Summary

The effect of ingestion of DL-ethionine on the testis of the rat was investigated and the resulting lesions evaluated according to Leblond and Clermont's classification. This classification proved to be useful as a standard for comparison of the various lesions.

After 12 days of ethionine administration, arrest of spermatogenesis was found beyond the cap phase spermatids. Giant cells were formed by groups of cap phase spermatids. No changes in the interstitial tissue were observed at this stage. After 24 days, all seminiferous epithelium had disappeared and only Sertoli cells remained. Marked numerical hyperplasia of Leydig cells was observed at this stage.

REFERENCES

1. Alvizouri, M., and Warren, S. Effects of *DL*-ethionine on the pancreas and other organs. *A. M. A. Arch. Path.*, 1954, **57**, 130–137.
2. Kaufman, N.; Klavins, J. V., and Kinney, T. D. Testicular damage following ethionine administration. *Am. J. Path.*, 1956, **32**, 105–115.
3. Becker, V. Die chronische Aethioninvergiftung der Ratte. *Verhandlungen der Deutsche Gesellschaft f. Path.*, 1956, **40**, 247–252.
4. Maun, M. E.; Cahill, W. M., and Davis, R. M. Morphologic studies of rats deprived of essential amino acids. I. Phenylalanine. *Arch. Path.*, 1945, **39**, 294–300.
5. Maun, M. E.; Cahill, W. M., and Davis, R. M. Morphologic studies of rats deprived of essential amino acids. II. Leucine. *Arch. Path.*, 1945, **40**, 173–178.
6. Maun, M. E.; Cahill, W. M., and Davis, R. M. Morphologic studies of rats deprived of essential amino acids. III. Histidine. *Arch. Path.*, 1946, **41**, 25–31.
7. Schwartz, C.; Scott, E. B., and Ferguson, R. L. Histopathology of amino acid deficiencies. I. Phenylalanine. *Anat. Rec.*, 1951, **110**, 313–327.
8. Scott, E. B., and Schwartz, C. Histopathology of amino acid deficiencies. II. Threonine. *Proc. Soc. Exper. Biol. & Med.*, 1953, **84**, 271–276.
9. Scott, E. B. Histopathology of amino acid deficiencies. III. Histidine. *A. M. A. Arch. Path.*, 1954, **58**, 129-141.
10. Scott, E. B. Histopathology of amino acid deficiencies. V. Isoleucine. *Proc. Soc. Exper. Biol. & Med.*, 1956, **92**, 134–140.
11. Leblond, C. P., and Clermont, Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. In: Biology of the Testes. Part 1. Spermatogenesis and Sperm Maturation. MacLeod, J., (cons. ed.). *Ann. New York Acad. Sc.*, 1952, **55**, Article 4, pp. 548–573.
12. Goldberg, R. C.; Chaikoff, I. L., and Dodge, A. H. Destruction of pancreatic acinar tissue by *DL*-ethionine. *Proc. Soc. Exper. Biol. & Med.*, 1950, **74**, 869–872.

13. Koch-Weser, D., and Popper, H. Hepatic fibrosis produced by chronic ethionine feeding. *Proc. Soc. Exper. Biol. & Med.*, 1952, **79**, 34–37.

14. Simpson, M. V.; Farber, E., and Tarver, H. Studies on ethionine. I. Inhibition of protein synthesis in intact animals. *J. Biol. Chem.*, 1950, **182**, 81–89.

15. Farber, E.; Simpson, M. V., and Tarver, H. Studies on ethionine. II. The interference with lipide metabolism. *J. Biol. Chem.*, 1950, **182**, 91–99.

16. Sós, J.; Kemény, T., and Schnell, M. Genitale Veränderungen an männlichen Ratten verursacht durch partiellen Methioninmangel. *Acta physiol. hung.*, 1953, **4**, 211–218.

17. Adamstone, F. B., and Spector, H. Tryptophan deficiency in the rat. Histologic changes induced by forced feeding of an acid-hydrolyzed casein diet. *Arch. Path.*, 1950, **49**, 173–184.

18. Siperstein, D. M. The effects of acute and chronic inanition upon the development and structure of the testis in the albino rat. *Anat. Rec.*, 1920–1921, **20**, 355–391.

19. Bouin, P., and Garnier, C. Altérations du tube séminifère au cours de l'alcoolisme expérimental chez le rat blanc. *Compt. rend. Soc. de biol.*, 1900, **52**, 23–25. Cited by Siperstein.[18]

20. Warren, S., and Olshausen, K. W. Interstitial cell growths of the testicle. *Am. J. Path.*, 1943, **19**, 307–331.

21. Fitzgerald, P. J.; Hellman, L.; Weinstein, J., and Schimmel, R. The concentration, distribution, and excretion of radio-ethionine (S^{35}) in the rat on stock and protein-depleted diets—determined by radioactivity counting and radioautography. (Abstract) *Am. J. Path.*, 1954, **30**, 619–620.

22. Howard, R. P.; Sniffen, R. C.; Simmons, F. A., and Albright, F. Testicular deficiency: a clinical and pathologic study. *J. Clin. Endocrinol.*, 1950, **10**, 121–186.

23. Unna, K.; Singher, H. O.; Kensler, C. J.; Taylor, H. C., Jr., and Rhoads, C. P. Effect of dietary protein on liver riboflavin levels and on inactivation of estradiol by liver. *Proc. Soc. Exper. Biol. & Med.*, 1944, **55**, 254–256.

24. Shipley, R. A., and György, P. Effect of dietary hepatic injury on inactivation of estrone. *Proc. Soc. Exper. Biol. & Med.*, 1944, **57**, 52–55.

25. György, P. Inactivation of estrone by liver: assay method *in vivo* for dietary hepatic injury in rats. *Proc. Soc. Exper. Biol. & Med.*, 1945, **60**, 344–349.

26. Biskind, G. R., and Biskind, M. S. The nutritional aspects of certain endocrine disturbances. *Am. J. Clin. Path.*, 1946, **16**, 737–745.

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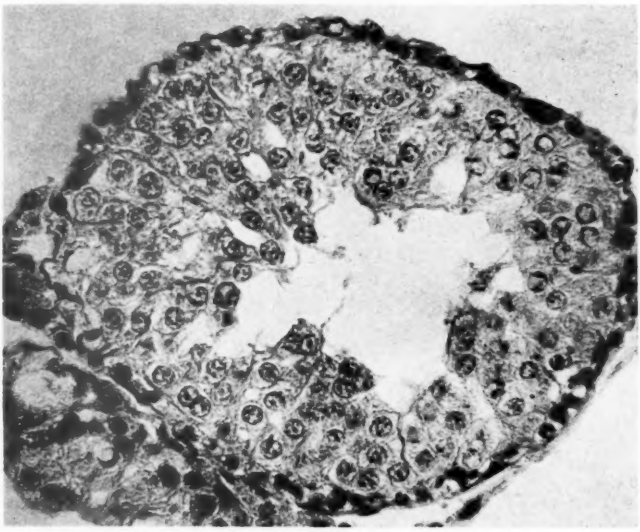
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Seminiferous tubule of rat in Golgi phase. Ethionine administered for 12 days. The predominant cell is a very young spermatid. Note absence of maturation phase spermatids and free spermatozoa. Periodic acid-Schiff (PAS) stain. × 450.

FIG. 2. Seminiferous tubule in cap phase. Ethionine administered for 12 days. The predominant cell has a capped nucleus. PAS stain. × 450.

FIG. 3. Seminiferous tubule in cap phase demonstrating pleomorphism of both nuclei and caps. PAS stain. × 450.



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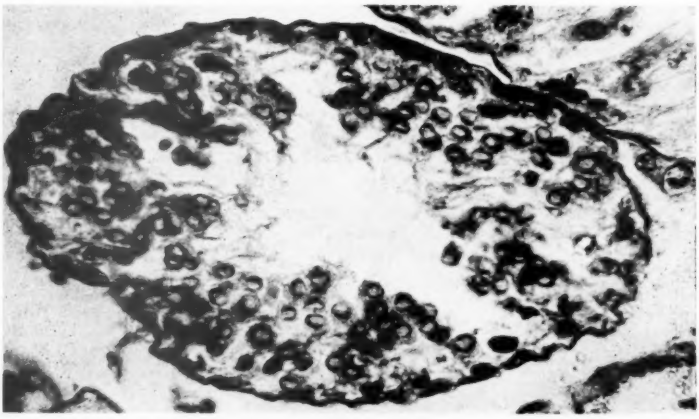

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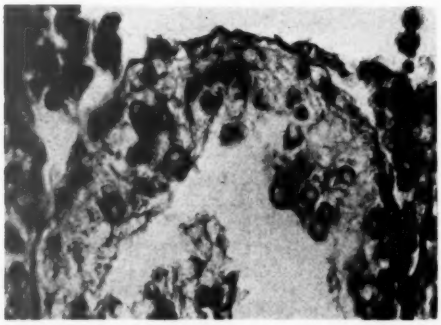

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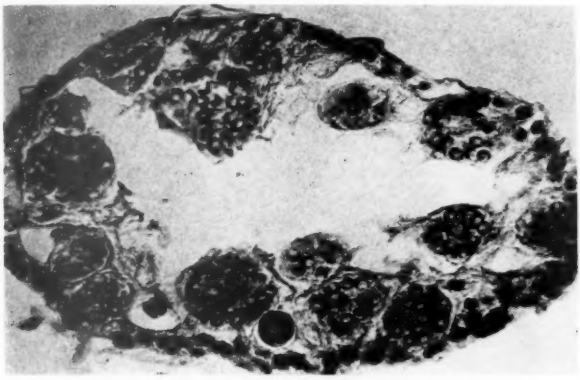
FIG. 4. Cap-nucleated giant cells in a seminiferous tubule. No other germinal epithelium is present. PAS stain. × 360.

FIG. 5. A cap phase seminiferous tubule. Giant cells contain capped nuclei which are scattered or appear at the periphery of the cytoplasm. Note vacuoles which represent the sites of degenerated giant cells. PAS stain. × 360.

FIG. 6. Seminiferous tubule in acrosome phase. Ethionine administered for 12 days. The peripheral layer of germinative cells is formed by leptotene and zygotene spermatocytes and several central layers of pacyhtene and diplotene spermatocytes. Immature and free spermatozoa are absent. Hematoxylin and eosin (H and E) stain. × 450.



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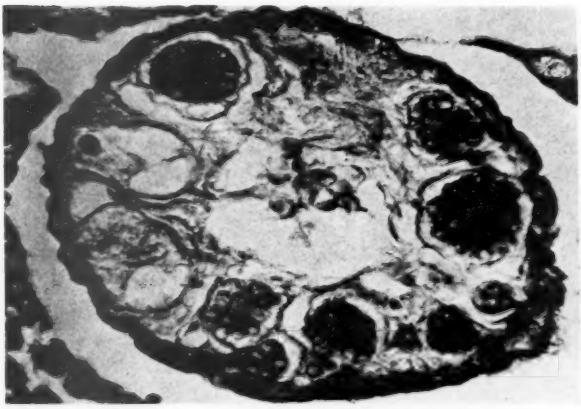

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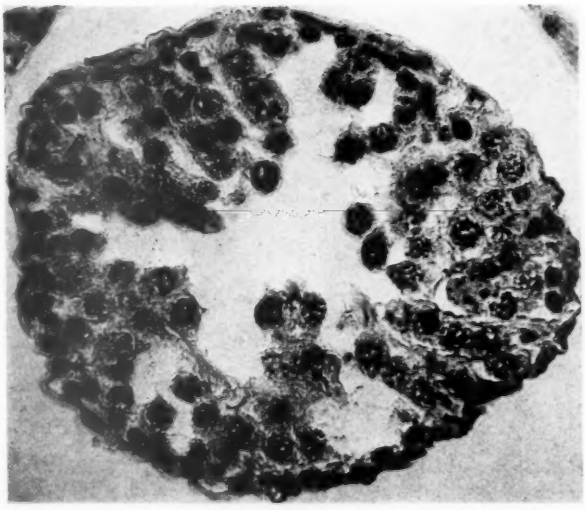

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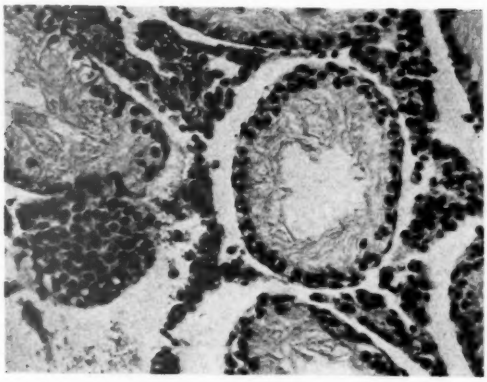
FIG. 7. Seminiferous tubule from a rat receiving ethionine for 24 days. Note atrophy of germinative epithelium and hyperplasia of Leydig cells. H and E stain. × 200.

FIG. 8. Seminiferous tubule of an untreated control rat. Animal is the same age as the rat serving in Figure 7. Spermatogenesis and tubule caliber are normal. The Leydig cells present a normal pattern. H and E stain. × 200.

FIG. 9. Liver of a rat receiving ethionine for 24 days. Note mild parenchymal damage. H and E stain. × 200.



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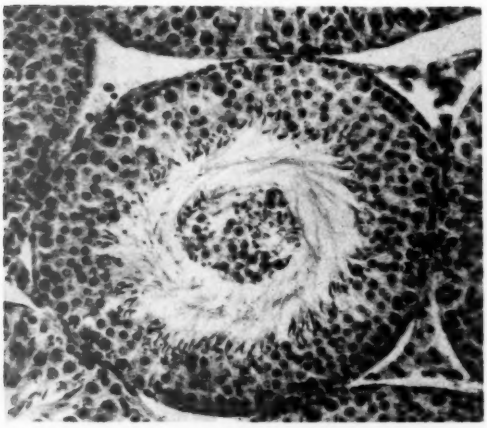

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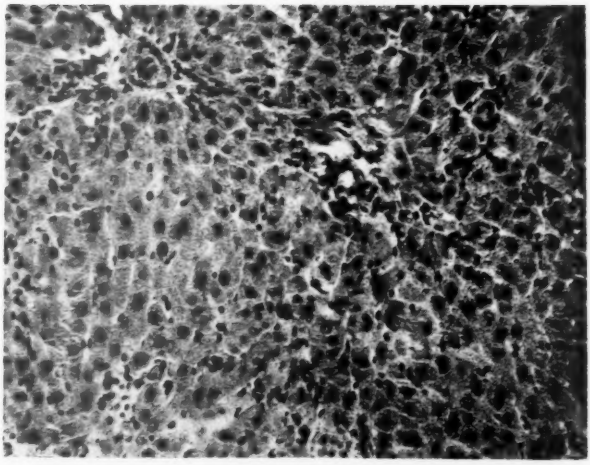

9

MYASTHENIA GRAVIS

II. Histochemical Demonstration of Acetylcholinesterase Activity in Motor End-plates of Extraocular Muscle in Patients with Myasthenia Gravis. A Post-mortem Study*

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Data are available on the quantitative determination of cholinesterase in the serum[1] and skeletal muscle[2] of patients with myasthenia gravis. Serum cholinesterase measured in these studies is not the acetylcholinesterase involved in impulse transmission at the myoneural junction and therefore does not reflect the activity of the specific enzyme at the neuromuscular junction. Quantitative determinations of acetylcholinesterase in striated muscle fail to provide a concept of activity at individual motor end-plates, and surely vary with the end-plate population of the muscle sample evaluated. The purpose of this investigation was to demonstrate acetylcholinesterase activity at motor end-plates of striated muscle. A histochemical method was utilized in order to compare this activity in muscles from patients with myasthenia gravis at necropsy with that in the muscle of non-myasthenic controls.

Material and Methods

The histochemical procedure used was the azo dye method described by Ravin, Zacks and Seligman.[3] In this method an artificial substrate, 6 brom-2-naphthyl acetate, is hydrolyzed by cholinesterases in the presence of a stabilized diazonium salt, resulting in precipitation of azo dye particles at the sites of enzyme activity. The technique utilizes the incubation of control sections in a solution of 10^{-6} eserine to distinguish cholinesterase from other forms of esterase activity. It was not necessary for the purpose of this study to further characterize the enzyme histochemically since the cholinesterase activity at the end-plate is largely acetylcholinesterase.[4,5]

Extraocular muscle was studied because end-plates are numerous in this tissue (Fig. 1). Although these were concentrated in relation to the ramification of the motor nerve, they could be found in all portions

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of the longitudinally sectioned muscle. In large skeletal muscles, which were originally investigated, end-plate populations varied considerably, depending on the proximity of the section to the point of maximum end-plate concentration. However, extraocular muscle could often be included in its entirety in a single block, thus insuring a fairly predictable population of end-plates for purposes of comparison. Extraocular muscle was affected clinically in all the myasthenic patients studied.

The muscles were obtained from 8 patients with myasthenia gravis and 10 control patients 3 to 5 hours after death. The existence of myasthenia gravis was established in each case by positive prostigmin tests, ergometry, and the existence of a typical clinical syndrome. The patients were all young or middle aged women whose thymus glands showed germinal center hyperplasia.[6] Seven patients died in respiratory paralysis, several shortly following thymectomy. The one well controlled patient died 24 hours after thymectomy as a result of massive pulmonary embolism. This patient's myasthenia appeared to be under good control at the time of death. Each patient was receiving large doses of prostigmin, often both orally and parenterally, at the time of death; several patients were receiving pyridostigmin in addition. The control cases were patients who had had neither neurologic nor muscle disease, and were receiving no drugs likely to affect acetylcholinesterase activity.

Results

The dye deposition indicative of enzyme activity was concentrated at the end-plates (Figs. 1 and 2) in the control specimens and in 5 of 8 specimens from myasthenic patients. The deposit was often so heavy that structural elements were obscured. The intensity of the reaction faded rapidly at short distances from the end-plates. In the muscle at a distance from recognizable end-plates, scattered granules of dye accumulated and tended to be deposited along cross striations. Muscle from 5 of the patients with myasthenia gravis who died of respiratory failure, failed to show appreciable acetylcholinesterase inhibition despite prostigmin therapy. These specimens appeared similar to the controls (Fig. 2). In the well controlled myasthenic patient who suffered a pulmonary embolus shortly after thymectomy, only minimal inhibition of enzyme activity was noted. Two myasthenic patients who died in respiratory failure showed marked inhibition of acetylcholinesterase activity. In the two patients in whom inhibition was manifest, there had been no significant differences in

terminal prostigmin dosage or clinical course when compared with the 5 cases which failed to show this phenomenon. The controls all exhibited uniformly high enzyme activity at the end-plates.

Discussion

The persistence of acetylcholinesterase activity in 5 myasthenic patients, in a degree indistinguishable from that encountered in the controls, despite large doses of prostigmin, is difficult to explain. It is not likely that the inhibitory effect of prostigmin is reversed in tissues 3 to 5 hours after death. Animals given sufficient prostigmin to inhibit end-plate acetylcholinesterase maintain the inhibition 24 hours post mortem.[7] It is probable that inhibition, once developed, would have persisted 3 to 5 hours in human tissues. In myasthenic crisis, prostigmin may in some way be prevented from inhibiting the acetylcholinesterase activity at the myoneural junction. This hypothesis is consistent with the occasional instance of clinical resistance to prostigmin observed in such crises. Recent histochemical evidence suggests that prostigmin given to a myasthenic patient who is not in crisis will inhibit acetylcholinesterase activity at the end-plates.[8] On the other hand, in our group of cases, two patients, clinically refractory to prostigmin, showed marked inhibition of acetylcholinesterase activity. Resistance to prostigmin therapy, even in the face of inhibition of acetylcholinesterase, may result from a block at the myoneural junction due to accumulation of acetylcholine. The possibility exists, however, that prostigmin may function pharmacologically in myasthenia gravis through some other mechanism than its inhibition of acetylcholinesterase. In support of this possibility was the absence of appreciable inhibition of enzyme activity in an apparently well controlled patient dying of another cause. There is, in fact, no sound evidence to indicate that prostigmin exerts all of its beneficial effect in myasthenia by its anticholinesterase action. Other investigators have suggested that prostigmin has a direct action on the myoneural junction, independent of its inhibition of acetylcholinesterase.[9] Further evidence along these lines is provided by the fact that pyridostigmin, an effective drug in the therapy of myasthenia gravis, is a relatively weak acetylcholinesterase inhibitor, and most probably acts directly upon the myoneural junction.[10] The observations in the present study emphasize an absence of correlation between the demonstrable inhibitory effect of prostigmin on acetylcholinesterase activity at the myoneural junction and the pharmacologic control of myasthenia gravis. The data support the hypothesis that prostigmin may

also benefit the myasthenic patient by direct action on the myoneural junction, independent of its capacity to inhibit acetylcholinesterase. Instances in which patients are refractory to the drug may be attributable to failure of this direct action.

Summary

A histochemical study of acetylcholinesterase activity in the endplates of extraocular muscle in 8 patients with myasthenia gravis and in 10 control patients is presented. Five of 7 patients in myasthenic crisis failed to demonstrate evidence of inhibition of acetylcholinesterase activity despite the administration of large doses of prostigmin. One patient whose disease was well controlled died of postoperative pulmonary embolism. The muscle in this case showed little inhibition of acetylcholinesterase activity by prostigmin. The implications of these results are discussed.

REFERENCES

1. Wilson, A., and Stoner, H. B. Myasthenia gravis: a consideration of its causation in a study of fourteen cases. *Quart. J. Med.*, 1944, **NS 13**, 1–18.
2. Goodman, L.; Carlson, R. I., and Gilman, A. Muscle and blood cholinesterase in myasthenia gravis: case study. (Abstract.) *J. Pharmacol. & Exper. Therap,*. 1939, **66**, 15–16.
3. Ravin, H. A.; Zacks, S. I., and Seligman, A. M. The histochemical localization of acetylcholinesterase in nervous tissue. *J. Pharmacol. & Exper. Therap.*, 1953, **107**, 37–53.
4. Augustinsson, K-B. Cholinesterases. A study in comparative enzymology. *Acta physiol. scandinav.*, 1948, **15**, Suppl. 52, 1–182.
5. Koelle, G. B. The histochemical differentiation of types of cholinesterases and their localizations in tissues of the cat. *J. Pharmacol. & Exper. Therap.*, 1950, **100**, 158–179.
6. Castleman, B. Tumors of the Thymus Gland. In: Atlas of Tumor Pathology, Section V, Fascicle 19, 82 pp. Subcommittee on Oncology of the Committee on Pathology of the National Research Council, Armed Forces Institute of Pathology, Washington, D.C., 1955.
7. Bergner, A. D., and Durlacher, S. H. Histochemical detection of fatal anticholinesterase poisoning. *Am. J. Path.*, 1951, **27**, 1011–1021.
8. Woolf, A. L.; Bagnall, H. J.; Bauwens, P., and Bickerstaff, E. R. A case of myasthenia gravis with changes in the intramuscular nerve-endings. *J. Path. & Bact.*, 1956, **71**, 173–178.
9. Riker, W. F., Jr., and Wescoe, W. C. The direct action of prostigmine on skeletal muscle; its relationship to the choline esters. *J. Pharmacol. & Exper. Therap.*, 1946, **88**, 58–66.
10. Blaschko, H.; Bülbring, E., and Chou, T. C. Tubocurarine antagonism and inhibition of cholinesterases. *Brit. J. Pharmacol.*, 1949, **4**, 29–32.

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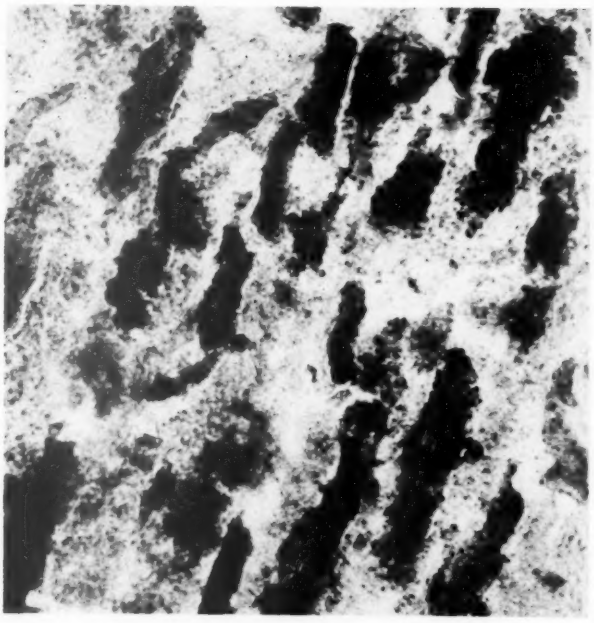
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Extraocular muscle from a normal patient, showing several end-plates. End-plate sites are represented by dense accumulations of azo dye particles. × 200.

FIG. 2. Muscle from a patient with myasthenia gravis. Detail of a motor end-plate stained for acetylcholinesterase activity. × 600.



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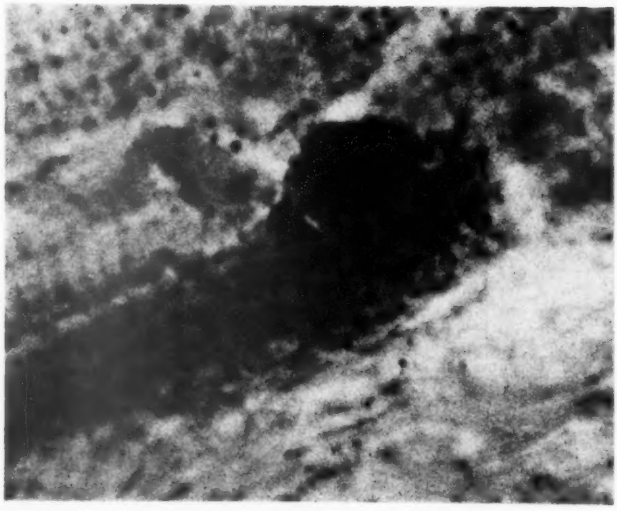

2

HISTOGENESIS OF BRANCHIAL CYSTS

A Report of 468 Cases*

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The majority of cystic lesions in the lateral aspect of the neck are believed to arise from remnants of the branchial arches and for this reason are called branchial cysts. Microscopically, it is seen that they have an epithelial lining, and their walls contain lymphoid elements of varying density.

In a recent article Bernier and Bhaskar[1] presented a classification of lympho-epithelial lesions of salivary glands, based on their investigation of 186 cases, which indicated that most of them arose from glandular inclusions within the lymph nodes of the parotid and cervical regions (Table I). In one member of this group (the "benign lympho-epithelial cyst") the epithelial inclusions gave rise to single or multiple epithelium-lined cysts. Although these cysts replaced the lymph nodes to varying degrees, they were always surrounded by lymphoid tissue. During the course of that investigation, it appeared, both clinically and microscopically, that the so-called branchial cyst closely resembled the "benign lympho-epithelial cyst" or the "cystic lymph node" of the parotid region. The present investigation was undertaken, therefore, to determine its histogenesis and natural history.

Review of Literature

It is generally believed that branchial cysts arise from the remnants of the branchial apparatus.[2-15] In 1913, however, Wenglowski[16] demonstrated that none of the adult structures below the hyoid bone originated from branchial clefts, and thus could not be derived from the branchial apparatus. He suggested, instead, an origin from the thymic duct. Meyer,[17,18] for the same reasons, agreed that all congenital anomalies arising from the branchial apparatus must be located in the region above the lower border of

Table I

Lympho-epithelial Lesions of Salivary Glands[1]

1.	Epithelial lesions arising in lymph nodes
	Cystic or multicystic lymph node (benign lympho-epithelial cyst)
	Papillary cystadenoma lymphomatosum
	Adenoma lymphomatosum
	Pleomorphic adenoma lymphomatosum
2.	Lesions primarily of lymphatic tissue in salivary glands
	Mikulicz's disease

* Received for publication, July 15, 1958.

the hyoid bone and that the branchial cysts arose from the pharyngothymic duct. King[5] reviewed the literature in 1949 and rejected the concepts of origin from branchial clefts, the precervical sinus, and the thymic duct. He concluded that branchial cysts arose in close association with the lymphatic tissue or lymph nodes; their epithelial lining originated from the endothelium of the nodes; and in some cases in which the supporting tissue of the cyst consisted of the thymus, the cyst lining was derived from Hassall's corpuscles.

Branchial cysts appear as circumscribed nodular swellings on the side of the neck. Most frequently they occur close to the anterior border of the sternocleidomastoid muscle.[5,9,10,14,15,19,20] The majority of them are seen in the upper third of the neck[21] or in association with the upper third of the sternocleidomastoid.[5,10,15,19,20] The incidence is greatest between 20 and 30 years of age, and apparently there is no relationship to sex.[5,19] The lesions vary in duration from 6 months to 10 years.[19] They vary in size from that of an olive to an orange[14] and in some instances fluctuate in size.[14]

These cysts are usually lined by stratified squamous epithelium,[5,7,14,15,19,21] pseudostratified ciliated columnar epithelium,[15] or epithelium of both types. It has been assumed that the epithelial lining is derived either from the ectoderm of the branchial clefts or from the entoderm of the pharyngeal pouches.[7,10,15] The cyst wall invariably contains varying amounts of lymphoid tissue.[5,7,10,14,15,17-19]

Branchial cysts are often confused with branchial fistulas. The two lesions, however, are distinct and separate.[10,20,21] Whereas the cysts do not have an opening into the pharynx and usually do not have an external sinus,[10] about 80 per cent of the fistulas communicate with the skin surface, and some have an opening or an extension to the pharynx.[21] The fistulas, unlike the cysts, are usually present at birth, are usually lined by columnar or ciliated epithelium, and exude mucus.[21]

Material and Methods

The present investigation is based on 468 specimens of branchial cyst at the Armed Forces Institute of Pathology. Sections were stained with Wilder's reticulum, hematoxylin and eosin, and Masson's trichrome stains.

Observations

Clinical Features

In our series, branchial cysts usually appeared as circumscribed, movable swellings which ranged from the size of a normal cervical lymph node to that of an orange (Figs. 1 and 2). The location of 295

lesions was not specified except that they occurred in the neck. Cases in which the exact location was given are summarized in Table II. In a number of instances the size of the cysts fluctuated. In some cases the onset of the swelling, and in others an increase in size, were associated with sore throat, common cold, upper respiratory or dental infection. In more than half (61 per cent) of the 468 patients, the cysts occurred between the ages of 21 and 30 years (Table III). The duration of the lesions ranged from 3 weeks to 10 years. Recurrences after excision were rare; however, if the treatment consisted of incision and drainage, the cystic swelling invariably recurred. In the present study almost 93 per cent of the lesions occurred in males, but since this material is derived almost exclusively from a predominantly male population, the sex incidence is biased.

TABLE II

Location of Branchial Cysts in 468 Cases

Location	No.
Neck	295
Angle of mandible	50
Anterior border of sternocleidomastoid	26
Below sternocleidomastoid	21
Submandibular area	18
Anterior triangle of the neck	14
Carotid triangle of the neck	12
Node on carotid artery	7
Posterior triangle of the neck	5
Preauricular area	5
Parotid area	5
"Lymph node":	
Neck	3
Carotid bifurcation	2
Anterior cervical chain	1
Internal jugular	1
Subclavicular	1
Postauricular	1
Superficial to sternocleidomastoid	1

Histologic Features (Table IV)

In 452 of the 468 cases the cysts were surrounded by lymphoid tissue and lined by stratified squamous epithelium which, in some instances, was keratinized. The epithelium was free of ridges. The lymphoid tissue was of both diffuse and follicular nature (Figs. 3 to 5). The boundary between the epithelium and lymphoid tissue was defined only by a basement membrane (Fig. 5). In 403 of the 452 specimens, the subcapsular or the medullary sinusoids, or both could be demonstrated in the lymphoid tissue (Figs. 6 to 8). The facility with which the sinusoids could be seen was often dependent upon the size of the cyst and the manner in which it involved the lymph node. If the cyst was large, the lymphoid tissue was compressed to a narrow peri-epithelial zone, and a careful search was necessary to demonstrate the sinusoids. In others, however, in which the cystic lesion had involved only part of the node, the nodal character of the lymphoid tissue was easily recognizable (Fig. 9). In these specimens various stages in the devel-

opment of branchial cysts were demonstrable, from small cysts with relatively unaltered lymph node structure to progressively larger cysts with less distinct nodal characteristics.

TABLE III

Distribution of Branchial Cysts According to Age

Age (yrs.)	Incidence (%)
0–10	2.3
11–20	13.2
21–30	61.0
31–40	16.0
41–50	5.0
51–60	1.2
61–70	1.3

TABLE IV

Histologic Features of 468 Branchial Cysts

Histologic features	No. of cases	Per cent
Lymphoid tissue in wall	452	96.6
Sinusoids in lymphoid tissue	403	86.1
Sinusoids not demonstrable	49	10.5
Stratified squamous epithelial lining	452	96.6
Periepithelial fibrosis	98	21.0
Cysts without lymphoid tissue	16	3.4

In 49 of the 452 specimens containing lymphoid tissue, sinusoids could not be seen. These lesions, however, were not cut serially and were represented by only one or two sections. In 98 lesions the zones immediately surrounding the epithelial lining showed some degree of fibrosis (Figs. 6, 9 and 10). In a few regions, collections of granulation tissue lay beneath the epithelium, which consisted of fewer cell layers than elsewhere in the cyst.

In 16 instances the branchial cysts were not associated with lymphoid tissue (Fig. 11). Of these, 2 were lined by respiratory epithelium, and in 1 case the stratified squamous lining contained numerous mucous cells.

DISCUSSION

Histogenesis of Branchial Cysts

It is a common belief that branchial cysts arise from remnants of the branchial clefts or pharyngeal pouches, although the principal evidence for this concept lies in the fact that the cysts occur in the general area of the gill arch apparatus. Another hypothesis concerning their origin is that they arise from the thymic duct. King[5] rejected these concepts, and suggested that since the lesions are closely associated with lymphoid tissue, they should be termed "lateral lymphoepithelial cysts." He did not, however, describe the mechanism of their formation.

The present investigation shows that at least 86 per cent and possibly 96 per cent of branchial cysts are, in reality, cystic lymph nodes.

This conclusion is based on the following observations: (1) The epithelial lining of the cysts is surrounded by lymphoid tissue in which both the subcapsular and medullary sinusoids can be demonstrated. (2) Epithelial inclusions are frequently seen in lymph nodes of the neck. A developing periparotid lymph node in a 3-month-old fetus is demonstrated in Figure 12. During the process of development, a periparotid lymph node might well include glandular elements within its substance. Similar epithelial inclusions have been described in the superficial and deep cervical nodes.[5] (3) Branchial cysts often fluctuate in size, and both enlargement and onset of pain are frequently associated with upper respiratory or dental infection. This would be expected if the cysts represent lymph nodes which drain such inflammatory sites.

The most favored view regarding the histogenesis of branchial cysts is that they arise from the remnants of the gill arch apparatus. Because of the following observations, such a possibility would appear unlikely. (1) Lesions identical to branchial cysts occur within the parotid gland. These have been shown to represent cystic lymph nodes.[1] Furthermore, since the parotid gland is not derived from the branchial arches, one cannot ascribe a branchiogenic origin to the cystic lesions in this location. (2) Appearance, exacerbation, or fluctuation in the size of the branchial cyst is often associated with a local inflammation, such as upper respiratory or dental infection. This clinical phenomenon is difficult to explain if one assumes a branchiogenic origin. (3) The presence of lymphoid tissue around these cysts cannot be explained on the basis of gill arch origin. (4) Branchial cysts, unlike branchial fistulas, are seldom, if ever, seen at birth. They occur most frequently after 20 years of age. (5) Branchial cysts are connected neither to the pharynx nor to the skin surface.

The thymic duct origin claimed by Wenglowski,[16] as well as by Meyer,[17,18] appears unlikely for reasons identical with those militating against branchiogenic origin. In addition, the observation that parathyroid or thymic tissue is never seen in the wall of the cyst would reflect against an origin from the thymic duct.

Whereas the larger majority of branchial cysts represent cystic lymph nodes, there remains a very small group which does not contain lymphoid tissue. In the present series this comprised less than 4 per cent of the lesions. Cystic cavities lined by squamous, cuboidal, ciliated columnar or mucus-secreting epithelium characterize such lesions, which appear to arise from the epithelial remnants left in the wake of organ migration in the cervical region or from the sequestration of skin

epithelium. The mechanism of development of cysts of this group, therefore, is analagous to that of the fissural cysts of the maxilla or epidermal cysts of the skin and mucous membranes.

Epithelial Inclusions in Lymph Nodes. It is quite apparent that a majority of branchial cysts stem from cystic alterations of epithelium trapped in cervical nodes, but the nature of this epithelium has never been investigated. The following possibilities exist: (a) the epithelium may be of branchial cleft origin; (b) it may represent the epithelium of some of the structures which arise from the pharyngeal pouches; and finally (c) it may be derived from the epithelium of the parotid gland.

The branchial clefts appear soon after the 26th day of fetal life and disappear between the 32nd and 45th days (5 to 15 mm. stages) to leave a smooth contour to the neck.[22] The cervical lymph nodes on the other hand begin development as aggregations of lymphoblasts after the second intra-uterine month, and they cannot be identified until after the third month *in utero*.[22] It would be highly improbable, therefore, that the epithelium seen in them is of branchial cleft origin.

Since the development of the cervical lymph nodes occurs at a time when the thyroid, parathyroid and thymus glands are still in the process of development, the inclusion of the epithelium of these regions in lymph nodes must be considered as a likely possibility. The only contradictory observation is the fact that no intranodal lesions in which the thyroid, parathyroid or thymic tissue is clearly identifiable have been observed.

The parotid gland begins its development at about the 45th day *in utero* (16 mm. stage) as a blind epithelial cord just posterior to the angle of the mouth. This cord of cells extends laterally to the future pre- and subauricular areas where in the third month of intra-uterine life rapid and repeated branching of the duct system occurs. The parotid gland now consists of lobules of ducts, separated by mesenchyme. At the stage during which the proliferation of the parotid duct system occurs, the cervical lymph nodes also develop. Many of these replace parotid lobules and incorporate glandular tissue within their substance. Since at this time the fetus does not have a well-defined neck, the developing parotid gland is in close proximity not only to the parotid lymph nodes but also to the upper cervical nodes. There is, therefore, a likely probability that the epithelium within cervical lymph nodes is of glandular origin. This assumption is supported by the observations that (a) salivary gland tumors arise in this epithelium and papillary cystadenoma lymphomatosum may be seen in a cervical

node far removed from any salivary gland[1]; and (b) so-called branchial cysts are most frequently seen in the upper third of the neck, an area where parotid tissue inclusions are most likely to occur.

Nomenclature

The term "branchial cyst" is used to imply a specific embryologic origin for these cystic lesions. We have seen, however, that they probably arise from epithelial inclusions within cervical nodes. It would, therefore, be appropriate to refer to them as ***benign cystic lymph nodes,*** or ***benign lympho-epithelial cysts.***

Clinical Features

The present investigation is in agreement with previous reports that the greatest number of branchial cysts occur between the ages of 21 and 30 years (Table III). The causes for the proliferation of the previously dormant epithelium in the cervical nodes at this period of life are obscure. However, the high incidence of these lesions at approximately 20 years of age might indicate some relationship to sexual maturity.

From observations in our series it would appear that the branchial cysts cannot be properly treated by drainage and aspiration but require complete enucleation.

SUMMARY

This investigation is based on a review of clinical and histologic observations in 468 specimens filed as branchial cysts at the Armed Forces Institute of Pathology. The study showed the following:

The branchial cysts do not arise from the remnants of the branchial apparatus. About 96 per cent of so-called branchial cysts in reality represent cysts in cervical lymph nodes. The cystic alteration occurs in the epithelium which is trapped in nodes of the neck during embryogenesis. In view of their histogenesis they should be termed "benign cystic lymph nodes" or "benign lymphoepithelial cysts."

Only 3 to 4 per cent of the branchial cysts do not have lymphoid tissue in their walls. These lesions probably arise in the misplaced epidermis or the epithelial remnants left in the wake of organ migration.

The majority of branchial cysts become clinically evident between 20 and 30 years of life.

If aspirated or drained, the branchial cysts recur; if excised they do not.

REFERENCES

1. Bernier, J. L., and Bhaskar, S. N. Lymphoepithelial lesions of salivary glands: classification and histogenesis. *Cancer,* 1958, 11, 1156–1179.
2. Heusinger, 1864. Quoted by King.[5]

3. Von Langenbeck, B., 1855. Quoted by King.[5]
4. Cusset, 1877. Quoted by King.[5]
5. King, E. S. J. The lateral lympho-epithelial cyst of the neck ("branchial cyst"). *Australian & New Zealand J. Surg.*, 1949, **19**, 109–121.
6. Bland-Sutton, J. Tumours, Innocent and Malignant. Their Clinical Characters and Appropriate Treatment. Cassell & Co., London, 1922, ed. 7, pp. 554–560.
7. Willis, R. A. The Principles of Pathology. C. V. Mosby Co., St. Louis, 1950, p. 646.
8. Lederer, F. L. Diseases of the Ear, Nose, and Throat. Principles and Practice of Otorhinolaryngology. Clinical Technics and Procedures. F. A. Davis Co., Philadelphia, 1952, ed. 6, p. 1139.
9. Andreassen, M. Congenital cysts and fistulas of the neck. *Acta oto-laryng.*, 1953, Suppl. **109**, 15–19.
10. Kinder, C. H. Branchial cyst and lateral cervical fistula. *Brit. J. Surg.*, 1954–1955, **42**, 53–56.
11. Thoma, K. H. Oral Pathology. A Histological, Roentgenological, and Clinical Study of the Diseases of the Teeth, Jaws, and Mouth. C. V. Mosby Co., St. Louis, 1954, ed. 4, p. 939.
12. Bernier, J. L. The Management of Oral Disease. A Treatise on the Recognition, Identification, and Treatment of Diseases of the Oral Regions. C. V. Mosby Co., St. Louis, 1955, p. 507.
13. Thoma, K. H., and Robinson, H. B. G. Oral and Dental Diagnosis, with Suggestions for Treatment. W. B. Saunders Co., Philadelphia, 1955, ed. 4, p. 116.
14. Proctor, B. Lateral vestigial cysts and fistulas of the neck. *Laryngoscope*, 1955, **65**, 355–401.
15. Ash, J. E., and Raum, M. An Atlas of Otolaryngic Pathology. Armed Forces Institute of Pathology, Washington, D.C., 1956, p. 423.
16. Wenglowski, R. Ueber die Halsfisteln und Cysten. *Arch. klin. Chir.*, 1913, **100**, 789–892.
17. Meyer, H. W. Congenital cysts and fistulae of the neck. *Ann. Surg.*, 1932, **95**, 1–26; 226–248.
18. Meyer, H. W. True branchiogenic cyst and fistula of the neck. *Arch. Surg.*, 1937, **35**, 766–771.
19. Sedgwick, C. E., and Walsh, J. F. Branchial cysts and fistulas. A study of seventy-five cases relative to clinical aspects and treatment. *Am. J. Surg.*, 1952, **83**, 3–8.
20. Hunt, C. J. Tumors of the neck of embryonic and epithelial glandular origin. *J. Internat. Coll. Surgeons*, 1955, **23**, 298–304.
21. Bailey, H. The clinical aspects of branchial fistulae. *Brit. J. Surg.*, 1933, **21**, 173–182.
22. Hamilton, W. J.; Boyd, J. D., and Mossman, H. W. Human Embryology. (Prenatal Development of Form and Function.) Williams & Wilkins Co., Baltimore, 1952, ed. 2, pp. 169; 178; 188; 197.

The authors wish to acknowledge the excellent technical assistance given by Miss Eliza Buddo in the preparation of histologic specimens used in this study. The photomicrographs were prepared by M/Sgt. Walter F. Engler of the Armed Forces Institute of Pathology Photographic Division.

[*Illustrations follow*]

LEGENDS FOR FIGURES

Except where indicated otherwise, sections illustrated in photomicrographs were stained with hematoxylin and eosin.

FIG. 1. "Branchial cyst" of 3 months' duration from the anterior border of the sternocleidomastoid of a 22-year-old white male. Gross specimen. × 1.5.

FIG. 2. Cut surface of the specimen shown in Figure 1. × 1.5.

FIG. 3. A periparotid cystic lymph node ("branchial cyst"). × 16.

FIG. 4. "Branchial cyst." The lymphoid tissue is separated from the lining epithelium only by the basement membrane, and the lesion is encapsulated. (AFIP Neg. 57-17651.) × 17.

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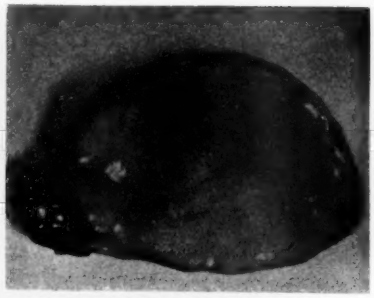

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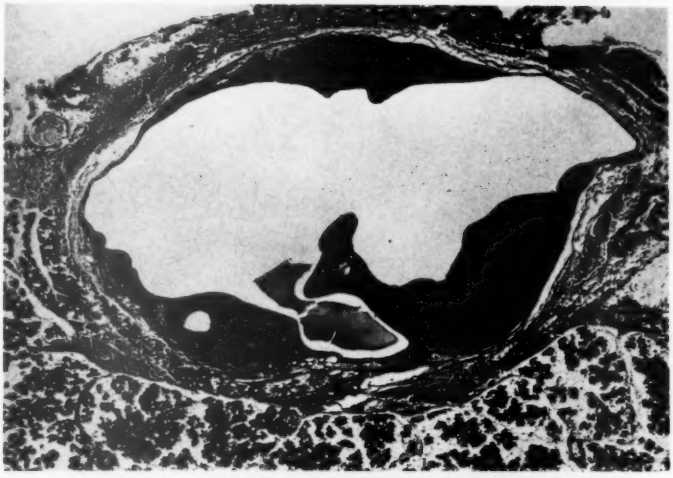

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FIG. 5. "Branchial cyst," showing epithelial lining, lymphoid tissue and medullary sinusoids. (AFIP Neg. 57-17642.) × 50.

FIG. 6. Low power photomicrograph of the wall of a "branchial cyst." The blocked areas are shown in higher magnification in two succeeding figures. (AFIP Neg. 57-17650.) × 16.

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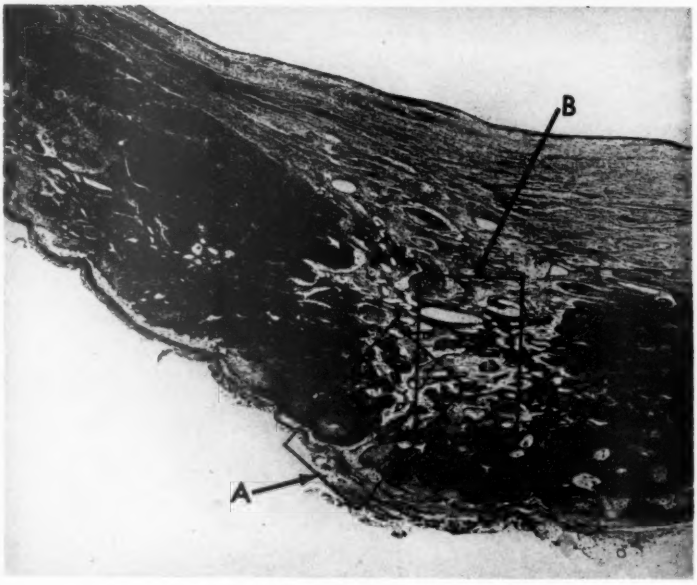

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FIG. 7. High power view of area (A) shown in Figure 6. Note the capsule and subcapsular sinus. (AFIP Neg. 57-17647.) × 50.

FIG. 8. High power view of area (B) shown in Figure 6. Note the medullary sinusoids. Wilder's reticulum stain. (AFIP Neg. 57-17646.) × 50.

FIG. 9. "Branchial cyst" which does not involve a part of the lymph node. The lymph node characteristics are therefore retained. (AFIP Neg. 57-17645.) × 13.

FIG. 10. "Branchial cyst," showing epithelial lining, zone of fibrosis, and numerous medullary sinusoids. (AFIP Neg. 57-17643.) × 50.

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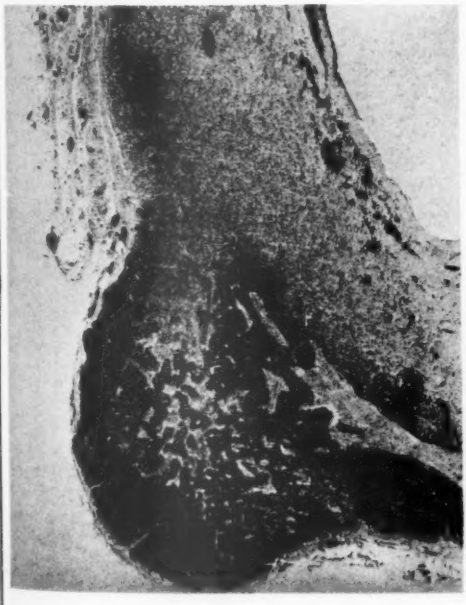

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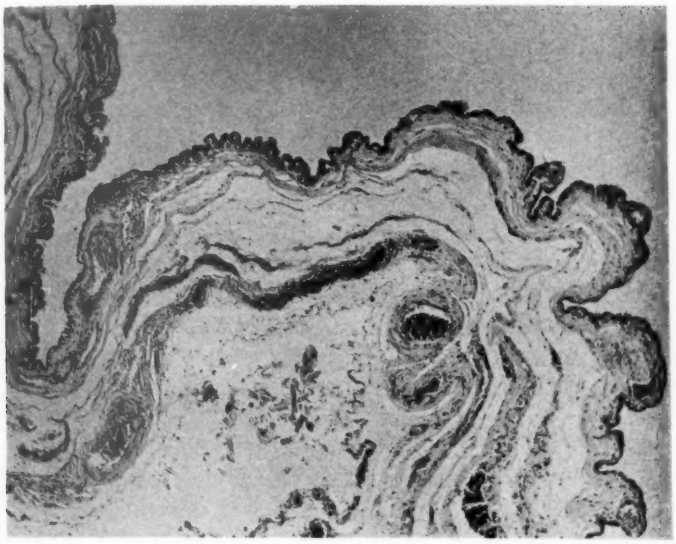
FIG. 11. "Branchial cyst" which is lined by columnar epithelium and does not contain a lymphoid component. (AFIP Neg. 57-17656.) × 50.

FIG. 12. A periparotid lymph node in a fetus. Note the epithelial inclusions in the node. (AFIP Neg. 57-16239.) × 100.



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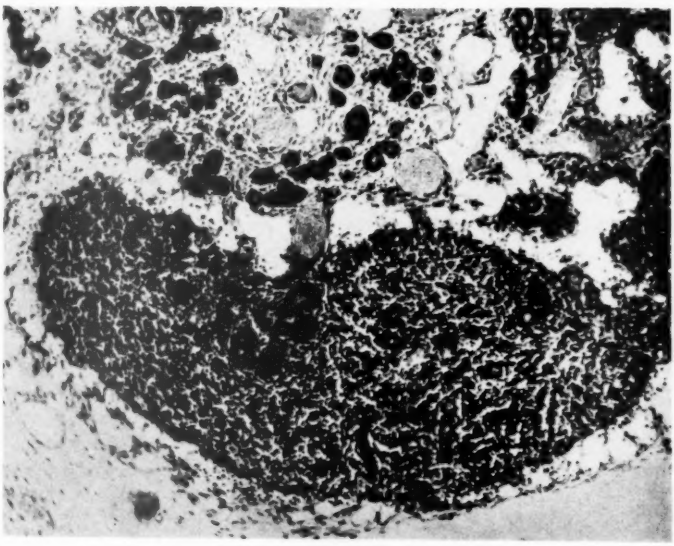

12

STUDY OF FUNCTIONAL ACTIVITY OF ADRENOCORTICAL CELLS WITH ELECTRON MICROSCOPY *

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The microscopic structure of adrenal cortex after stimulation by ACTH, stress, and other means has been studied extensively.[1,2] It is known that as the adrenal cortex produces increased cortical steroid hormones, there is a reduction of lipid within the cytoplasm. Anisotropic substance, presumably cholesterol, decreases or disappears, the vitamin C content is diminished, and the stainable ribonucleic acid in the cytoplasm is increased in amount. The cells of the zona fasciculata, initially predominantly clear and pale-staining, become compact and dark-staining. The adrenal glands in Cushing's disease with diffuse cortical hyperplasia also exhibit a marked increase in the number and prominence of dark-staining, granular cells in the zona fasciculata.[3] In an attempt to ascertain the ultrastructural aspects of this change, we have made electron microscope studies of the adrenocortical cells of the rat after the administration of ACTH.

METHODS

Forty-five young adult male albino rats weighing from 175 to 250 gm. were utilized. Control animals were sacrificed by rapidly fracturing the cervical vertebrae, and the adrenal glands were procured within 60 seconds. One group was given ACTH subcutaneously in a dosage of 5 units, and the adrenal glands examined 4 hours later, after sacrifice by the same method as the control group. In the third group, ACTH was administered daily from 7 to 14 days, and the adrenals were procured in a similar manner.

Small (1 mm.) blocks of adrenal tissue were fixed in the customary manner in chromium-osmic acid at pH 7.4 (Dalton's fixative) for electron microscopy.[4] Other specimens were prepared by immediate freezing with CO_2 on a Spencer freezing microtome. Before the tissue had thawed, full slices at 50 μ thickness were sectioned. The slices were passed rapidly through Tyrode's solution, and then into the Dalton fixative. In all cases, the tissues were carried through alcohol

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for dehydration, and embedded in methacrylate. The flat disc method of Borysko[5] was used, and under the microscope the 50 μ slices were cut into small blocks, making it possible to obtain cells from any desired portion of the gland. Adrenal specimens were also embedded in paraffin, and conventional sections stained with hematoxylin and eosin were examined under the light microscope. Oil red O stain for detection of lipid was also utilized in frozen sections.

Results

Our observations were made specifically upon the cells in the zona fasciculata, where the major changes associated with the functional status are known to occur. In sections from normal controls, with light microscopy, the outer portion (approximately half) of the cortex was always composed of cells containing abundant lipid droplets which appeared as clear vacuoles in paraffin sections (Figs. 1 and 2), and stained intensely with oil red O in frozen sections. The cells in the deeper portion of the fasciculata were dark-staining in the normal adrenal, and contained little lipid.

In animals sacrificed 4 hours after the administration of ACTH, a definite cytoplasmic alteration occurred; dark-staining, compact cells now extended throughout most of the zona fasciculata (Figs. 3 and 4). In some instances a persistent outer zone of cells contained lipid, but this was decreased in amount. Very few small droplets of lipid were visible in the deeper portion of the zona fasciculata. The cytoplasm in most of the components of the fasciculata was compact, granular and eosinophilic. In animals treated with ACTH for a week or more, a similar but more marked increase in granular, dark-staining cells was evident in the zona fasciculata.

Preparations of adrenal cortex which had been rapidly frozen with CO_2 and sectioned at 50 μ before thawing, and the small blocks fixed in Dalton's solution revealed essentially the same electron microscopic appearance. There was no marked cytoplasmic distortion by ice crystal formation; this was possibly attributable to the resiliency of unfixed cytoplasm and restoration of its structure with thawing before fixation. In tissue which had been frozen, cytoplasmic constituents were slightly separated, but mitochondria and other organelles seemed to be well preserved. It was noted that tissue prepared in this manner was invariably well fixed, and well stained with osmic acid. The lipid was usually represented only by vacuoles, presumably because the minute sections had been passed through alcohol; some vacuoles still contained rims of osmiophilic material. Generally, tissue fixed in this manner yielded better ultrathin slices than block-fixed material.

With electron microscopy, the pale-staining, lipid-filled cells of the outer portion of the normal adrenal cortex contained numerous, large, clearly defined vacuoles, representing the lipid droplets observed with the light microscope. In block-fixed tissue, these appeared as homogeneous, osmic acid-stained, rounded masses (Figs. 5 to 7). The cytoplasm contained well defined mitochondria, although they were separated from one another by variable numbers of lipid droplets. The number of mitochondria per unit volume of cytoplasm was, therefore, not great. Some of these were compact or dark, containing small vesicles and double-membraned profiles; others had a looser, granular, and non-vesicular internal structure. The endoplasmic reticulum was of a finely granular and filamentous nature, although in a few regions small vesicle formation could be seen throughout the cells.

The dark-staining, granular cells of the outer portion of the zona fasciculata, which appeared following ACTH stimulation, exhibited a somewhat different appearance with electron microscopy (Figs. 8 to 10). Lipid droplets were markedly reduced in size and number. Mitochondria were noticeably larger, and more numerous. They were closely packed in many areas, and generally occupied a greater amount of the total area of cytoplasm. The mitochondria varied in electron density, but they were filled strikingly with vesiculated membranes. Some mitochondria were quite dense, apparently containing osmium-stained substance. Smaller, dense, osmium-stained bodies without definite mitochondrial internal structure were also found. In ACTH stimulated adrenals, the outer mitochondrial membranes were frequently observed to be polylaminated, as noted by Lever.[6] In some of the glands that had been stimulated by ACTH for a week or more, the outer mitochondrial membranes were very indistinct, and there seemed to be a free continuity between the endoplasmic reticulum and the internal mitochondrial structure. The endoplasmic reticulum seemed to be increased in amount and showed a markedly vesicular appearance. Almost all of the reticulum was converted into continuous cords and clusters of vesicles which were larger and more numerous than in normal, unstimulated glands.

In the nuclei of ACTH stimulated cortical epithelium, the nuclear membrane often exhibited a multi-laminated appearance. There was also some suggestion that the endoplasmic reticulum could be traced in continuity with the outer laminas of the nuclear membrane. Since the endoplasmic reticulum contains most of the ribonucleic acid content,[7] this phenomenon could conceivably represent the method of increased formation of ribonucleic acid shown to occur in ACTH stimulated adrenocortical cells.

The dark-staining cells in the deeper portion of the normal zona fasciculata and zona reticularis resembled those in the outer portion of the fasciculata of the adrenal cortex after the administration of ACTH. They contained a vesicular endoplasmic reticulum, vesicular mitochondria, and were essentially similar in other respects.

In the normal adrenal cortex, as in the adrenal stimulated by ACTH, a striking relationship could be seen between the endoplasmic reticulum and the lipid droplets. A few more or less complete double membranes around the perimeter of the fat droplets seemed to be produced by the endoplasmic reticulum. Also, in many lipid droplets, small fringes of vesicles projected slightly into the lipid vacuole. This suggested the transformation of lipid droplets into cytoplasmic organelles, and the possible formation of mitochondria in this manner.

Discussion

Lever[6,8] previously described the ultrastructure of the adrenal cortex in the rat and hamster. His observations were specifically in reference to the mitochondria, which, he noted, had a vesicular structure in the deeper portion of the zona fasciculata. He, too, observed alterations in the cells following ACTH stimulation. The vesicular composition of endoplasmic reticulum and of mitochondria has also been described in other cell types and in different animal species.[9]

The increased prominence of vesicular structure in the cytoplasmic reticulum and mitochondria in conjunction with lipid depletion following the administration of ACTH strongly suggests that this phenomenon is a feature of increased cellular activity and the cytosynthesis of cortical steroids. The rapid increase in the number of mitochondria at the time of reduction in number of lipid droplets, and the appearance of double membranes and vesicles at the periphery of the vacuoles suggest that lipid droplets may be transformed into mitochondria as the functional activity of the cell is increased. One may speculate that these lipid droplets in the course of conversion represent areas where cholesterol is undergoing intermediate metabolic alteration, leading to the formation of cortical hormone.

Since it is possible that the increase of vesicular endoplasmic reticulum and mitochondria signifies increased hormone synthesis, and since the cells of the outer portion of the zona fasciculata of the normal gland have less vesicular cytoplasmic reticulum and fewer mitochondria than those in the deeper regions, it is conceivable that the major sites of hormone synthesis are in the deeper portions of the zona fasciculata and reticularis.

Summary and Conclusions

The outer portion of the zona fasciculata in the normal adrenal contains cells which are pale-staining and filled with lipid. The ultrastructure of these cells is characterized by finely membranous and moderately vesiculated endoplasmic reticulum and mitochondria. The vacuolated cells under ACTH administration become dark-staining and lipid-depleted. In these, electron microscopy demonstrates an increase in endoplasmic reticulum and the development of a marked vesicularity. Their mitochondria are increased in size and number. This structural modification is thought to be related to cytosynthesis of adrenocortical steroids from cholesterol. The existence of a similar vesicular endoplasmic reticulum and an abundance of mitochondria in the cells of the deeper portion of the zona fasciculata and zona reticularis in the normal adrenal suggests that these are the major sites of synthesis of adrenocortical hormone under normal conditions. With stress or ACTH stimulation, hormone synthesis is probably increased by the enhancement of individual cell activity, and by the assumption of increased function in cells of the outer zona fasciculata. As new mitochondria are formed in these more active cells, lipid droplets disappear. Evidence is presented that suggests incorporation of lipid droplets into newly formed mitochondria. There is also an indication that the endoplasmic reticulum may stem from the outer lamellas of the nuclear membrane.

References

1. Symington, T.; Duguid, W. P., and Davidson, J. N. Effect of exogenous corticotropin on the histochemical pattern of the human adrenal cortex and a comparison with the changes during stress. *J. Clin. Endocrinol.*, 1956, **16**, 580–598.
2. Greep, R., and Deane, H. Cytology and cytochemistry of adrenal cortex. *Ann. New York Acad. Sc.*, 1949, **50**, 596–615.
3. Ashworth, C. T., and Garvey, R. F. The diffuse adrenal lesion in Cushing's disease. *Am. J. Path.*, 1958, **34**, 1161–1171.
4. Dalton, A. J. A chrome-osmium fixative for electron microscopy. (Abstract.) *Anat. Rec.*, 1955, **121**, 281.
5. Borysko, E. Recent developments in methacrylate embedding. II. Methods for the sectioning of optically selected single cells, the orientation of the plane of sectioning and the identification of the region of the specimen included in the sections. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, No. 4, Suppl., 15–20.
6. Lever, J. D. Physiologically induced changes in adrenocortical mitochondria. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, No. 4, Suppl., 313–318.
7. Palade, G. E., and Siekevitz, P. Liver microsomes. An integrated morphological and biochemical study. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, 171–200.
8. Lever, J. D. Electron microscopic observations on the adrenal cortex. *Am. J. Anat.*, 1955, **97**, 409–429.
9. Palade, G. E. The endoplasmic reticulum. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, No. 4, Suppl., 85–98.

LEGENDS FOR FIGURES

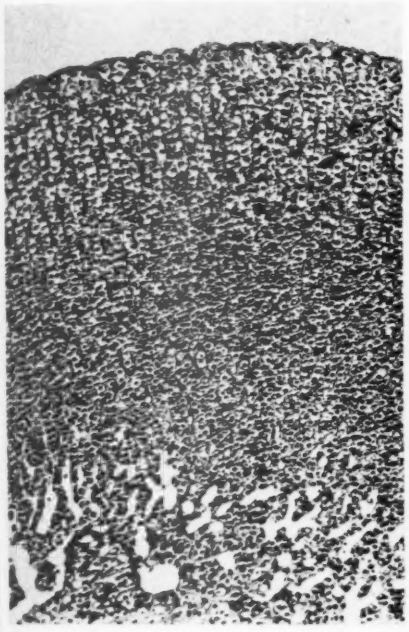
FIG. 1. Adrenal cortex of normal control rat. The outer portion of the zona fasciculata contains prominent, large, clear, intracellular lipid vacuoles. Hematoxylin and eosin (H and E) stain. × 116.

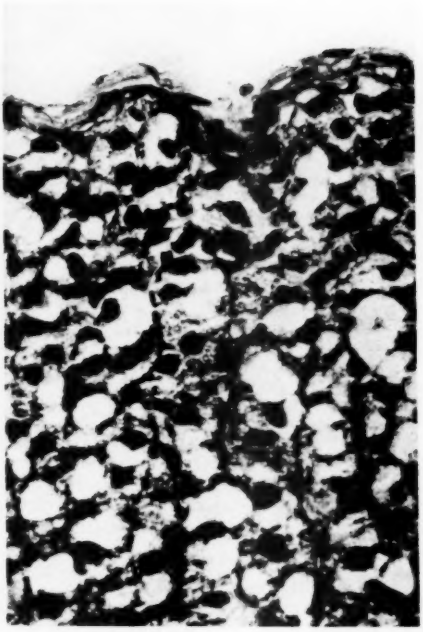
FIG. 2. Higher magnification of normal adrenal cortex, showing the zona glomerulosa and the outer portion of the zona fasciculata. Abundant lipid vacuoles are noted in the cells of the fasciculata. H and E stain. × 600.

FIG. 3. Adrenal cortex of rat receiving ACTH for two days. The cells of the zona fasciculata are enlarged and exhibit a compact, granular, and dark-staining cytoplasm. Lipid vacuoles are not visible at this magnification. H and E stain. × 116.

FIG. 4. High power view of the glomerulosa and outer part of the zona fasciculata of the adrenal shown in Figure 3. The compact, dark-staining, granular cytoplasm of the cells in the zona fasciculata is evident. Very small lipid vacuoles are visible. H and E stain. × 600.

The light microscopy photographs were prepared by the Medical Arts Department of the University of Texas Southwestern Medical School.





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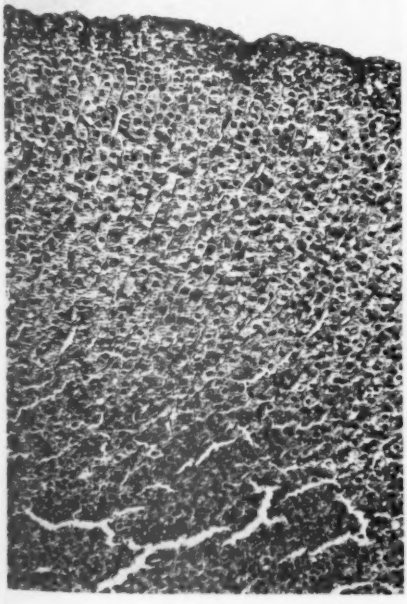

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FIG. 5. Electron photomicrograph of the outer portion of the zona fasciculata. Normal rat. Numerous lipid vacuoles (L) are seen. The mitochondria (M) are compact and widely separated. The endoplasmic reticulum (ER) is finely granular, and vesicles, though present, are quite small and indistinct at this magnification. Tissue prepared by fixation of 50 μ frozen slices in chromium-osmium tetroxide. × 6,700.

FIG. 6. Electron photomicrograph of the outer portion of the zona fasciculata. Normal rat. Some mitochondria (M) contain vesicular internal membranes. The endoplasmic reticulum (ER) also contains some fine vesicles. Lipid vacuoles are encompassed by finely vesicular membranes. Tissue prepared by small block fixation in chromium-osmium tetroxide. × 14,000.

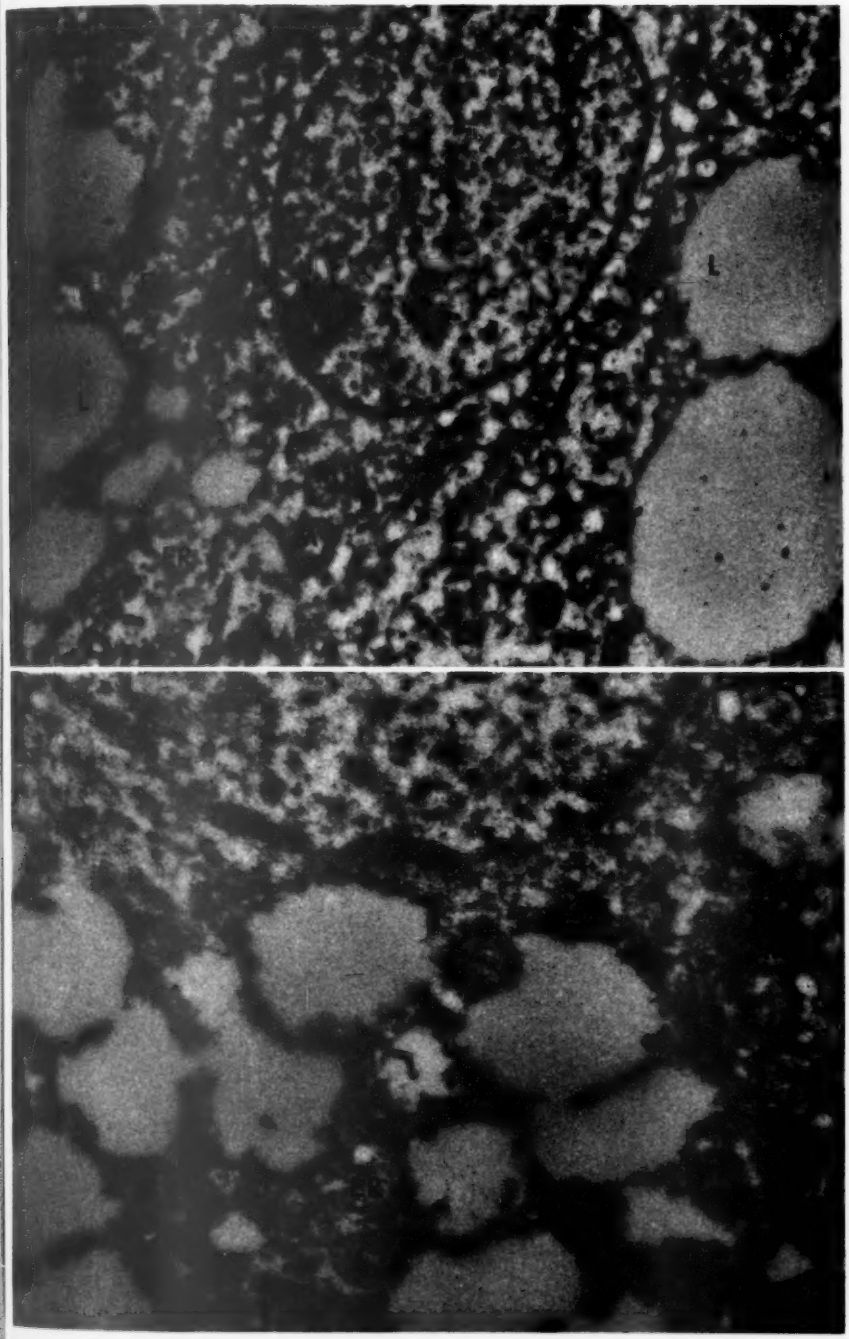

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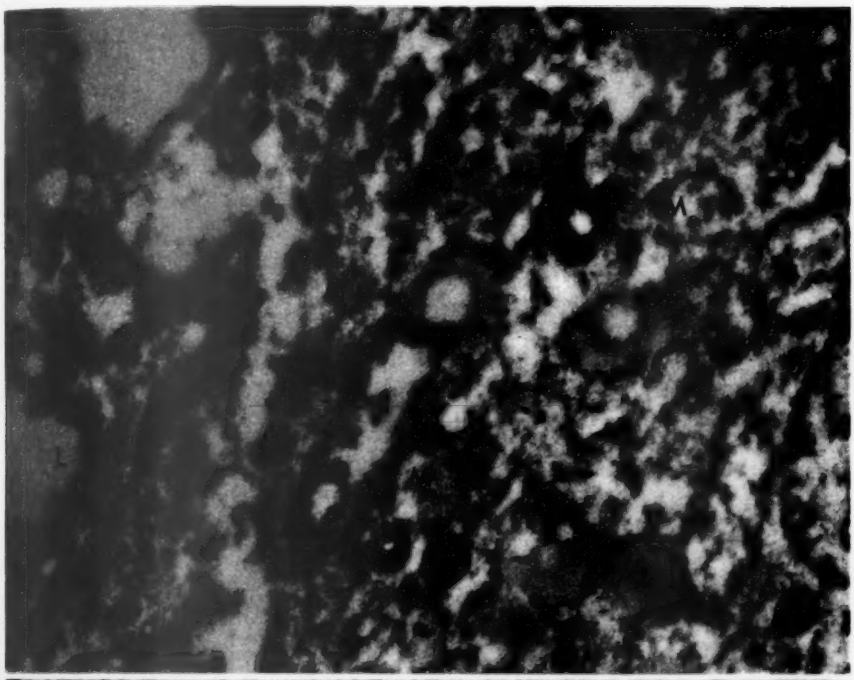
FIG. 7. Electron photomicrograph of the outer portion of the zona fasciculata. Normal rat. In one cell (a) the lipid vacuoles (L) are large, and all lipid has been dissolved in preparation. In another cell (b), the lipid droplets (L) are smaller and still contain osmium-stained fat. In this cell the mitochondria (M) are more numerous than in (a). Tissue prepared by small block fixation in chromium-osmium tetroxide. × 14,000.

FIG. 8. Electron photomicrograph of the outer portion of the zona fasciculata. Rat received daily injections of ACTH for two days. Tissue obtained 4 hours after the last injection. Lipid vacuoles have almost completely disappeared. Mitochondria are larger and vary from dark osmiophilic bodies (M_1) to larger and more loosely textured ones (M_2). Vesicles are very prominent in the mitochondria. The endoplasmic reticulum (ER) is notably vesicular in structure. A dark, small, osmiophilic body (B) is evident. The sinusoids contain red cells (S). Tissue prepared by osmium tetroxide fixation of 50 μ frozen section slices. × 6,700.



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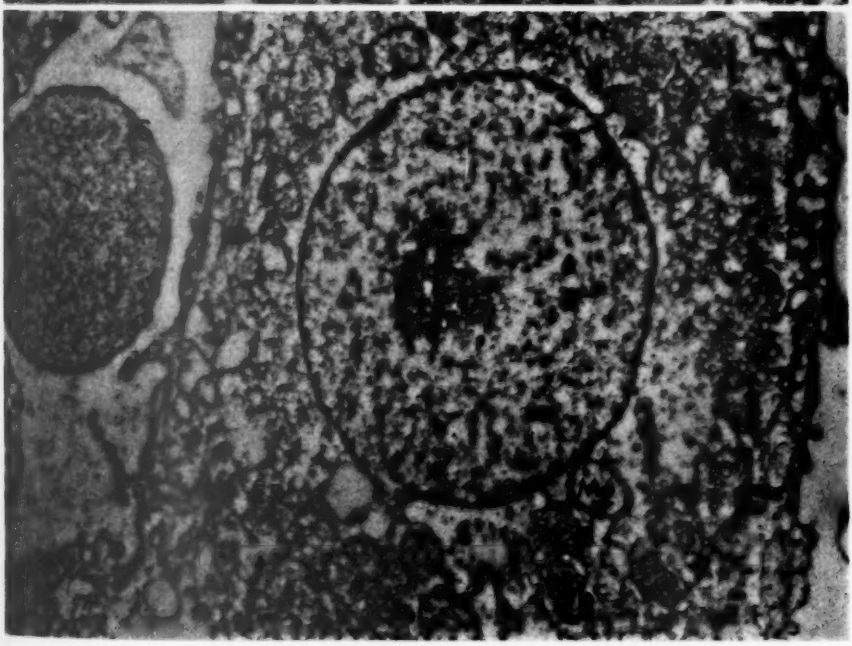

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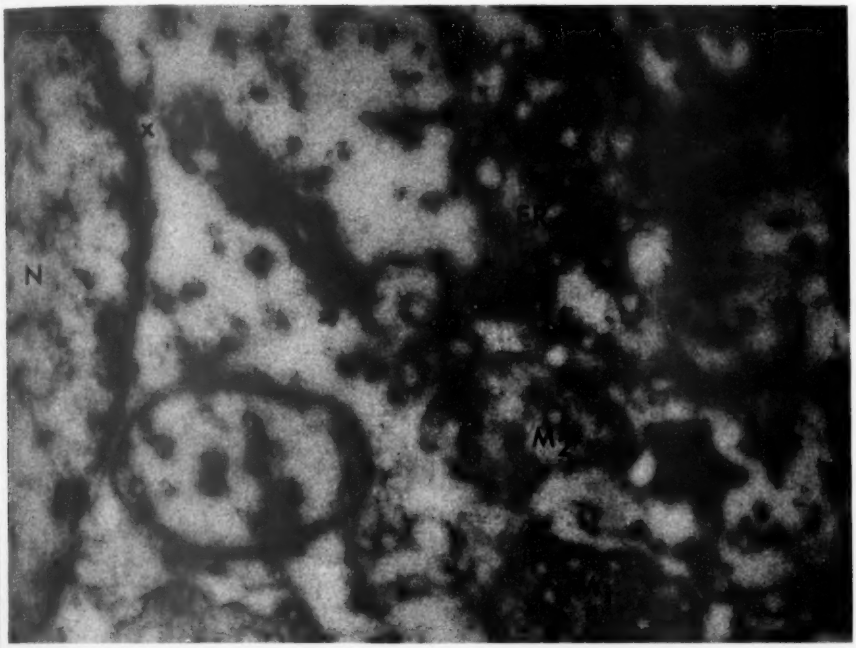
FIG. 9. Electron photomicrograph of a cell in the outer portion of the zona fasciculata. Rat received 2 daily injections of ACTH, the last one 4 hours before sacrifice. A portion of the nucleus is shown (N). The variation in compactness and density of mitochondria (M_1, M_2) and the vesicular nature of the endoplasmic reticulum (ER) are shown. A dark, stellate, osmiophilic body may be seen at the lower right. Continuity of endoplasmic reticulum with the nuclear membrane is suggested by the appearance at "X." Tissue prepared by chromium-osmium tetroxide fixation of 50 μ frozen slice. × 22,500.

FIG. 10. Electron photomicrograph of a cell in the outer portion of the zona fasciculata. Rat received daily injections of ACTH for one week. An abundant, highly vesicular endoplasmic reticulum (ER) is apparent. Outlines of mitochondria are poorly delineated; discernible mitochondria (M) are sparse. A small portion of a lipid droplet (L), most of which has been removed in preparation, is shown. Tissue prepared by small block fixation in chromium-osmium tetroxide. × 22,500.



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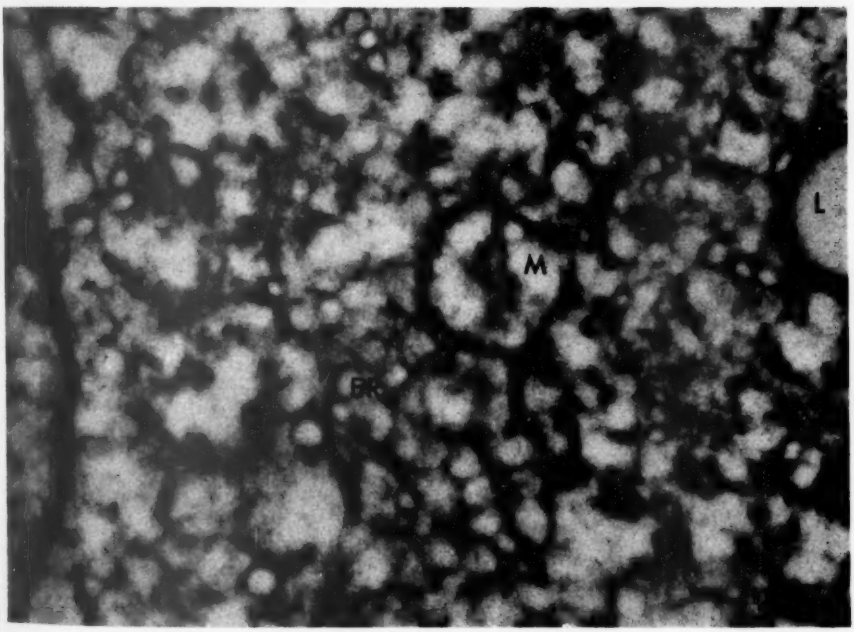

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